frontiers | Research Topics

Genetically engineered products: Preparing for the future

Edited by
Patricia Machado Bueno Fernandes, Deise Maria Fontana Capalbo and Carmen Vicien

Published in
Frontiers in Bioengineering and Biotechnology

ISSN 1664-8714
ISBN 978-2-83250-967-8
DOI 10.3389/978-2-83250-967-8

About Frontiers

Frontiers is more than just an open access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers journal series

The Frontiers journal series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the *Frontiers journal series* operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

Genetically engineered products: Preparing for the future

Topic editors

Patricia Machado Bueno Fernandes — Federal University of Espirito Santo, Brazil
Deise Maria Fontana Capalbo — Brazilian Agricultural Research Corporation (EMBRAPA), Brazil
Carmen Vicien — University of Buenos Aires, Argentina

Citation

Fernandes, P. M. B., Capalbo, D. M. F., Vicien, C., eds. (2022). *Genetically engineered products: Preparing for the future*. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-83250-967-8

Table of contents

frontiers | Frontiers in Bioengineering and Biotechnology

TYPE Editorial
PUBLISHED 22 November 2022
DOI 10.3389/fbioe.2022.1077237

OPEN ACCESS

EDITED AND REVIEWED BY
Ranieri Cancedda,
Independent Researcher, Genova, Italy

*CORRESPONDENCE
Patricia Machado Bueno Fernandes,
patricia.fernandes@ufes.br

SPECIALTY SECTION
This article was submitted to Biosafety and Biosecurity,
a section of the journal
Frontiers in Bioengineering and Biotechnology

RECEIVED 22 October 2022
ACCEPTED 28 October 2022
PUBLISHED 22 November 2022

CITATION
Fernandes PMB, Vicien C and Capalbo DMF (2022), Editorial: Genetically engineered products: Preparing for the future.
Front. Bioeng. Biotechnol. 10:1077237.
doi: 10.3389/fbioe.2022.1077237

Editorial: Genetically engineered products: Preparing for the future

Patricia Machado Bueno Fernandes[1]*, Carmen Vicien[2] and Deise Maria Fontana Capalbo[3]

[1]Biotechnology Core, Federal University of Espírito Santo, Vitória, Brazil, [2]Faculty of Agronomy, University of Buenos Aires and Institute for Scientific Cooperation in Environment and Health, Buenos Aires, Argentina, [3]Brazilian Agricultural Research Corporation (EMBRAPA), Brasília, Brazil

KEYWORDS

GM regulation, public perception, risk management, CRISPR, bioengineering

Editorial on the Research Topic
Genetically engineered products: Preparing for the future

The benefits of first-generation GM crops are remarkable and could be greater if there had been wider adoption of these technologies. Looking back, many of the constraints, discussions, and difficulties observed in the registration and commercialization of GM crops were based on regulatory structures and risk analysis topics. On one side, the regulatory structure varied from country to country and on the other side, the varied requirements made it difficult to have GM products approved. Due to this, only private companies were economically prepared to reach the market.

However, new technologies such as gene editing proved to be more specific, faster, and predictable, and, mainly, had lower regulatory costs. Therefore, they can be developed for the market by small companies and research institutes and may contribute to major environmental policy initiatives as many products under development in plants, animals, and microorganisms are designed to provide specific environmental benefits. Nevertheless, this would require researchers and developers of gene-edited products to have a clearer understanding of the regulatory landscape and how a product moves from early development to commercialization.

All this leads to the main objective of this Research Topic, which seeks to undertake a brief retrospective examination of the positive and negative effects of GM materials, mostly considering the relationship between regulation and innovation, with specific attention to gene editing techniques. Aspects related to public perception and communication were also taken into account. This would allow us to envisage the future.

With a retrospective look at 30 years of regulatory submission data, George et al. try to understand and forecast how the new SECURE rule from APHIS in the US might affect future diversification trends. In a more recent case, Vesprini et al. present some important modifications enacted during 2020 and 2021 in Argentina's regulatory policies on the Environmental Risk Assessment (ERA), thus exploring the possibilities of introducing

novel approaches to enhance the ERA and make it more efficient by applying scientific criteria and the accumulated experience and scientific bibliography on the Research Topic. Rocha-Salavarrieta also brings an important case from Latin American countries, related to regulatory harmonization. This harmonization is, in itself, a government responsibility, since governments define, implement, and are responsible for what and how to regulate. Harmonization can be aided by aligning definitions, standardizing the information needed to make informed decisions, defining timeframes for making determinations, and contemplating the possible recognition of decisions made by other countries. This article describes how those Research Topics can be addressed in a cooperative way, by neighboring countries, to effectively contribute to safe biotechnology development.

Gene-edited products bring an opportunity for the creative adaptation of the current regulatory regimes, to learn from the experience of the safe use of GM technologies, and allow for the opening of innovation opportunities beyond the limited range of basic crops. In a review of CRISPR/CAS- and topical RNAi-based technologies for crop management and improvement, Távora et al. address several aspects related to risk assessment, toxicity, and advances in the use of these tools. For Argentina's regulatory system, Goberna et al. examine how regulatory management took advantage of scientific progress to boost innovation and give more opportunities to local developers. Dealing with the uncertainties and risks of new genomic techniques, another publication, from Bouchaut et al., shows results from five workshops based on one case (genetic engineering of plants' rhizosphere) trying to identify tensions between different stakeholder groups. The authors propose a tool—a script on how to organize a stakeholder workshop—using anticipatory strategies to lower or mitigate uncertainties, helping to identify knowledge gaps as well. Jordan et al. report the findings from interviews and deliberative workshops from a broad multi-sector deliberative group and consider the merits of gene editing relative to alternative plant-breeding methods as a means for improving crops for Continuous Living Cover (CLC) agriculture, which they consider a powerful tool for developing and expanding to scale. In this sense, Fernandes et al. discuss how the long-overdue partnership between biotechnology and organic agriculture is fundamental for the mitigation of food insecurity and is a way forward to truly sustainable agriculture. They point out that if regulatory hurdles are not unfeasible, CRISPR technology and its derived seeds will be viable for small family farmers and could be the basis of sustainable organic agriculture.

Another Research Topic is that of consumer concerns; being well known that public opinion is ambivalent or critical towards foods derived from GM materials. Therefore, Collazo et al., address attitudes of the Ecuadorian University Community toward GM organisms based on socio-demographic variables, knowledge, beliefs, practices, and bioethical approach, indicating an incipient acceptance of GM organisms in the academic sector that might corroborate a transformation in the thinking of Ecuadorian civil society.

More traditional aspects of the environmental effects of GM products are reviewed and analyzed in order to discuss what and how new technologies could benefit their risk-benefit balance, using previous GM studies. Seixas et al. review and discuss the environmental effects due to pesticides for two different GM seeds, insect-resistant cotton and herbicide-tolerant soybeans, in a particular period of Brazilian agriculture from 2009–2013, using a dataset on commercial farms' use of pesticides and biotechnology. Horizontal gene transfer (HGT), i.e., the acquisition of genetic material that has not been inherited from a parent, assessments utilizing new tools for detection as well as next-generation sequencing are presented by Philips et al. Their discussion leads to an updated view of the likelihood, factors, and barriers to the occurrence of HGT in a variety of recipients, using mainly the framework of the Australian legislation.

As bioengineering advances, Gemler et al. describe the need for a biohazard review, shifting from organism-based analyzes to function-centered classifications. They present a new methodology for classifying biohazards at the individual sequence level, which they have compiled to distinguish the biohazard property of pathogenicity at the whole genome level. The resulting database can be used to develop hazardous "fingerprints" based on the functional metadata categories. The authors foresee that such a shift could lead to the improvement and standardization of current biosecurity and biosafety practices.

In conclusion, this Research Topic provided a multidisciplinary view of GMO regulation, focusing on relevant aspects of politics, economics, agronomics, health, and the safety of GE products. It covers a wide range of articles and reviews within the field, grouping a series of results with impacts and potential benefits of GE products to society, food/feed chains, and the environment.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Funding

PF acknowledges the Conselho Nacional de Desenvolvimento Científico e Tecnológico—CNPq and Fundação de Amparo à Pesquisa do Espírito Santo—FAPES for the research productivity awards (grants # 308306/2021 and 269).

Acknowledgments

We deeply thank all the authors and reviewers who have participated in this Research Topic.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

frontiers in Bioengineering and Biotechnology

PERSPECTIVE
published: 31 January 2022
doi: 10.3389/fbioe.2021.834589

Update of Argentina's Regulatory Policies on the Environmental Risk Assessment

*Facundo Vesprini *, Agustina Ines Whelan, María Florencia Goberna, Mariana Lucía Murrone, Gabriela Evangelina Barros, Andrés Frankow, Perla Godoy and Dalia Marcela Lewi*

National Directorate for Bioeconomy, Ministry of Agriculture, Livestock and Fisheries, Buenos Aires, Argentina

The Environmental Risk Assessment (ERA) of genetically modified (GM) crops in Argentina is carried out by the National Advisory Commission on Agricultural Biotechnology (CONABIA) and the Innovation and Biotechnology Coordination (ClyB). Both have a large experience with this assessment, since 1991, when CONABIA was created. The continuous support to biotechnology as a state policy and as part of the decision to encourage developers in the regulatory process has helped make progress in the revision of the regulations. The experience gained during the last 30 years and the worldwide scientific advances supported the bases to update the regulatory framework. Focusing on the biosafety strengthening and the improvement of the applicant's experience in the GM crops evaluation process, during 2020 and 2021, the ERA went through a reviewing process. Some important modifications were made, such as (i) the assessment of stacked GM crops with focus on the possible interactions between transgenes and the expression products, (ii) the strengthening of the ERA taking into account the transportability of data and conclusions from the Confined Field Trials (CFTs), (iii) the adoption of Familiarity and History of Safe Use (HOSU) concepts on the risk assessment of the expression products, (iv) the special considerations for the unintended effects of insertional sites, and (v) as a post commercial release of GM crops, the Insect Resistance Management Plan (IRMP) was reformulated. These novel approaches enhance the ERA; they make it more efficient by applying the science criteria and the accumulated experience and scientific bibliography on the topic.

Keywords: GM crop, data transportability, stacked GM crops, unintended effects, history of safe use, environmental risk assessment, familiarity, insects resistant management plan

OPEN ACCESS

Edited by:
Deise Maria Fontana Capalbo, Brazilian Agricultural Research Corporation (EMBRAPA), Brazil

Reviewed by:
Jeff Wolt, Iowa State University, United States
André Nepomuceno Dusi, Brazilian Agricultural Research Corporation (EMBRAPA), Brazil

****Correspondence:***
Facundo Vesprini
fvesprini@magyp.gob.ar

Specialty section:
This article was submitted to Biosafety and Biosecurity, a section of the journal Frontiers in Bioengineering and Biotechnology

Received: *13 December 2021*
Accepted: *27 December 2021*
Published: *31 January 2022*

Citation:
Vesprini F, Whelan AI, Goberna MDF, Murrone MLD, Barros GE, Frankow A, Godoy P and Lewi DM (2022) Update of Argentina's Regulatory Policies on the Environmental Risk Assessment. Front. Bioeng. Biotechnol. 9:834589. doi: 10.3389/fbioe.2021.834589

INTRODUCTION

Argentina was one of the first countries to have a regulatory framework for genetically modified (GM) crops for agricultural use. The evolution of the Environmental Risk Assessment (ERA) in Argentina is based on the updating of the regulations for different activities with GM crops as science advances and the experience accumulated. Argentine regulations have been in force and running since the early 1990s and take into account the criteria and considerations established in the Cartagena protocol and other international treaties. At the time that the National Advisory Commission on Agricultural Biotechnology (CONABIA) and the Innovation and Biotechnology Coordination (CIyB) decided to work on updating the regulations that contemplate the requirements for the commercial authorization of GM crops from the environmental point of view of agro-

ecosystems, different issues involved in the risk assessment were identified. These different issues were considered and treated in order to simplify the regulatory process of these products. It should be noted that the main aim in the updating process is biosafety. The purpose of this paper is to describe the evolution of this process and how the regulations for different topics were developed and updated. These topics were data transportability, stacked GM crops, Familiarity and History of Safe Use (HOSU), unintended effects, and Insect Resistance Management Plan (IRMP). Despite the fact that assessment by similar constructions is not described in this review, the criterion was ratified. It is based on GM crops with similar constructions that share the same characteristics of interest using the same molecular mechanisms to other commercial GM crops. The assessment criterion is based on establishing the absence of new or increased risks with respect to the previously assessed GM crop. Additionally, risk assessments are framed from the application of an analysis system based on the Problem Formulation (PF). Under this consideration, risk hypotheses that are identified linked the crop, the new phenotype, and its interaction with the agro-ecosystem, with focus on biosafety.

UPDATED PROCESSES ON REGULATORY POLICIES

Assessment of Stacked GM Crops

Stacked GM crops refer to conventional breeding crossing single GM crops containing individual transgenes with single or multiple traits. Single GM crop is defined as the insertion of DNA into the plant genome as a result of a single transformation process (Pilacinski et al., 2011). Many of the stacked GM crops contain insect and herbicide tolerance traits for controlling a broad range of insect pests and weeds (Que et al., 2010). Each single GM crop must have gone through the ERA and have a safety conclusion to apply at the stack assessment.

In the beginning of the stacked GM assessments, each application was considered as a new GM, and it went through the full assessment as a single GM crop. Therefore, all the molecular, phenotypic, and the interaction between the stack GM crop and the environment had to be presented. With the accumulated experience and based on the problem formulation approach, referring to analyze and verify risk hypotheses considering the weight of evidence, the assessments have gone through a simplification process, where redundant information related to each single GM crop was left aside. Using conventional breeding to combine GM crops does not involve insertion of new recombinant DNA sequence into the genome and does not modify the existing genomic DNA (Pilacinski et al., 2011).

From the above review process, applying the PF approach and considering the case-by-case assessment, the CONABIA and the CIyB decided that the assessment of stacked GM crops must focus on the possibility of interaction between novelty traits and genes. It was one of the most relevant topics of the resolution 32/2021 from the Secretary of Food, Bioeconomy, and Regional Development. The potential of interactions in the stacked GM crops is based on an understanding of the mode of action of the transgenes and their products (Kramer et al., 2016), specifically the possibility of epistasis between introduced genes or interaction between expression products in related metabolic pathways. At the same time, specific data to verify the absence of interaction, when supported by a risk hypothesis, became relevant, for example, to verify the absence of synergism between insecticide proteins. If it exists, a new non-target organism study must be done with the combinations of proteins. As a result of this interaction assessment, the risk for the environment of planting the stacked GM crop is analyzed.

Transportability of Data and Conclusions From the Confined Field Trials

In the ERA for the commercial release of GM crops, the CONABIA and the CIyB have applied some complementary approaches about the transportability of data and conclusions from CFT. CFTs are based on a comparative agro-phenotypic assessment between transgenic and non-transgenic (usually the isogenic or a near-isogenic line) with the aim to identify any differences between the GM crop and its non-GM comparator resulting from the intended or unintended consequences of the genetic modification (García-Alonso et al., 2014; Nakai et al., 2015). With these data, risk hypotheses are answered. CFTs involve plants grown side by side that are therefore subject to the same environmental conditions and agronomic management (Vesprini, et al., 2020). Data transportability builds on the premise that well-designed CFT may inform the ERA and support regulatory decision-making for GM plants being cultivated in another country (García-Alonso et al., 2014; Ahmad et al., 2016; Vesprini et al., 2020).

In the beginning of the Argentinean ERA, local CFTs were required and studies from other countries were considered as a weight of evidence to support the conclusion about the GM crop biosafety. Later, as García-Alonso et al. (2014) describes, foreign CFT replaced some local ones if they were done in similar agro-ecological conditions as the Argentinian crop production zone. This approach of data transportability comparing similar environments (climate, weather, and soil type) between regions to transport data became a useful tool to avoid redundant CFTs. At the same time, if the CFT is replicated in the country of interest, it is expected to have the same conclusion.

After years of ERA, it was evidenced that the conclusions arrived at in CFT that were analyzed in a wide range of environmental conditions can be transportable to other geographies, regardless of the agro-climatic and agro-ecological conditions (Vesprini et al., 2020). On this approach, the site selection with focus on the diversity of tested environments examined were key elements (Vesprini et al., 2020). The diversity selection of environmental conditions to perform the CFT is related to the crop production zone. At the same time, as the approach comparing agro-ecological conditions, the methodology and agronomic management of the studies and the measured endpoints are relevant to consider (Vesprini et al., 2020). If these three items are met, not only the data (as an informative study) but also the conclusions of the CFT are transportable. Therefore, if this study is performed again

considering other wide environments of the crop production zones, the conclusions arrived at will not change. This approach has specific considerations for risk hypotheses obtained through PF related to the GM crop and its interaction with specific environments. If any of these risk hypotheses needs a CFT in the site of interest, performing the CFT in that site may be justified. Otherwise, comparing agro-ecological conditions is a useful tool to analyze if the environmental conditions under concern were considered in a foreign CFT.

Familiarity and History of Safe Use

The CIyB and the CONABIA have carried out numerous ERA of different GM crops, repeatedly evaluating the same expression products. Moreover, both in Argentina and in many other countries, the commercial cultivation and consumption of crops expressing these expression products provide HOSU and support the conclusions reached by the CIyB and the CONABIA in the decision documents.

Recently, the CIyB updated the risk assessment process for GM Crops based on the familiarity and history of safe use (HOSU) of crops and expression products.

The concepts of HOSU are an integral part of PF, as the availability of existing information is a critical element that adds to the weight of evidence (Capalbo et al., 2020).

Familiarity is defined in the new Argentine guideline as "pre-existing scientific knowledge, experimental evidence, and accumulated regulatory experience on new expression products or on GM crops that can be taken into account in an ERA". Thus, the collection of documents, data, and existing literature constitute support material and form the weight of evidence for ERA.

Additionally, the HOSU is defined in the new Argentine guideline as the tradition in use, where scientific procedures or formal knowledge are not necessarily available or limited. However, given the history reported by the empirical evidence of use without adverse effects, it can be used as strong evidence to reach conclusions about the safety of new expression products, GM crops, or receptor crops. Both definitions have been supported in Capalbo et al. (2020).

By applying these two concepts, the main goal is to avoid redundancy of information declared in the different ERA applications.

All in all, in the new guideline, the applicant has the option to report if the expression products have familiarity or HOSU. In the event of no new or different information having emerged in relation to previous ERA performed for those expression products, it will be considered that the product has familiarity and/or HOSU.

However, it should be noted that, since the analysis is done on a case-by-case basis and is based on scientific/technical reviews, this measure will be ineffective if there is new information that invalidates the conclusions on which the previous opinions were based. Therefore, new relevant information must be presented and submitted to the CIyB and the CONABIA for consideration in order to carry out the analysis.

Unintended Effects of Insertional Sites

The CIyB and the CONABIA also updated the ERA for GM crops related to the unintended effects of insertional sites. During the genetic engineering transformation process, the DNA fragment of interest is inserted into the genome of a plant, often accompanied by additional DNA fragments and can also generate deletions and/or rearrangements. These genetic changes are collectively known as insertion effects and have the potential to give rise to unintended traits in plants (Schnell et al., 2015). These modifications could also alter genes or regulatory elements of the plant genome and generate new open reading frames (ORFs).

The relationship between genotype and phenotype in plants is complex and the role of the environment cannot be ignored. In many ways, plants are buffered against the consequences of genomic changes by the high level of genetic redundancy in their genome and by the quality control systems active in them. All of these factors influence whether or not an insert effect will produce an unintended characteristic (Schnell et al., 2015). Moreover, plant genomes are very dynamic, plastic, and undergo frequent insertions and other rearrangements (Ladics et al., 2015).

Glenn et al. (2017) conclude that extensive regulatory requirements have been established for GM crops, using a comparative safety assessment process. Thereafter, numerous studies have found transgenic varieties to be compositionally equivalent to conventional crops and that there are few exceptions of cases where the desired trait confers an intentional change in composition, such as improved nutrition. Moreover, the above-mentioned author states that global GM crop regulators have concluded over the past 20 years that, excluding GM crops with an intentionally improved composition, all evaluated traits of commercialized GM crop varieties are equivalent to varieties with a history of safe use. This is, in part, the result of the same plant selection practices used by breeders to minimize unintended effects, whether arising from spontaneous genetic changes that occur during conventional breeding (Schnell et al., 2015) or from the use of biotechnology to insert DNA into the plant genome.

Both the new ORFs and flanking sequences could be analyzed by the data generated in the field assays. When these effects appear, the plants are discarded by the developers during the screening process of the different events, in the field, in the greenhouse, or in the laboratory (Privalle et al., 2012; Glenn et al., 2017). This way, the absence of unintended effects is confirmed by an adequate formulation of the risk hypothesis of the GM crop on the agro-ecosystem, and is answered by carrying out the agro-phenotypic characterization studies that include the analysis of different parameters such as germination power, seed latency, phenology, phenotype, and behavior against biotic and abiotic stresses, which are carried out in multiple sites that cover a wide variability of agro-climatic conditions.

Bearing these considerations in mind, the updated guideline only refers to the unintended effects caused by the insertional site, but other effects caused by different mechanisms are not considered. Additionally, the compositional analysis is always exhaustively assessed by the Food and Feed Safety Committee

(CTAUOGM) from the National Service of Agri-Food, Health and Quality (SENASA).

Taking this into account, the applicant has the option to complete a form explaining the unintended effects in relation to the risk of the GM crop on the agro-ecosystem, according to what has been observed in the agro-phenotypic studies.

Insect Resistance Management Plan

Evolution of resistance in insect populations is a natural process and agricultural practices are intended to delay or mitigate insect resistance management (IRM). The Argentine experience through the past years has shown that joint actions must be taken by all parties involved such as industry, growers, and governmental agencies (Signorini et al., 2018). One of the key measures for delaying the evolution of resistance is the implementation of a refuge area in a GM insect-resistant plot in addition to crop rotation, weed management, insect monitoring, and insecticide applications when pest populations reached economic thresholds in refuge and communication programs about the topics above.

The previous guideline (2014) was updated with several recommendations so as to improve this plan for the applicants and for the regulatory system. The changes made were as follows:

1. The IRPMs are presented only for those GM crops that are going to be commercialized (single or stack) in such a way to avoid unnecessarily presentations and information.
2. Optional models can be introduced. The computerized model gives information about the product life cycle. The percentage of refuge can be justified by other means, for instance, papers and other documents showing crops with the same proteins and pests.
3. Specific decision document for IRPM is concluded when the plan is evaluated and the agreement is given by CONABIA and CIyB previously to enroll the GM crop in the National Registry of Cultivars from the National Seeds Institute.
4. The results of the susceptibility baseline must be presented together with the IRPM, and those of the damage baseline must be presented within 2 years from the date of registration of the first cultivar in the National Registry of Cultivars.

CONCLUSION

During the ERA process, carried out by CONABIA and the CIyB, the PF methodology is applied, through the formulation of risk hypotheses of the GM crops on the agro-ecosystem. When this assessment concludes, a decision document with all relevant information of the analysis process is drawn up. This document reflects the conclusions on biosafety for the agro-ecosystem of the evaluated GM crops.

After 30 years of having started the regulatory path of GMOs (October 1991) and 25 years after the first approval of a commercial crop, Argentina has maintained a continuous process of improvement. The regulatory system has been proactive and dynamic, analyzing the dossiers on a case-by-case basis, based on science and maintaining high biosafety standards. This experience allowed CONABIA to be named as FAO's reference center in Biosafety since 2014. Following the path of continuous updating and improvement and addressing the new challenges that arise, a specific regulation for molecular farming is currently being addressed and other topics related to biosafety in different subjects such as plants, animals, and microorganisms for agriculture purposes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

REFERENCES

Ahmad, A., Negri, I., Oliveira, W., Brown, C., Asiimwe, P., Sammons, B., et al. (2016). Transportable Data from Non-target Arthropod Field Studies for the Environmental Risk Assessment of Genetically Modified maize Expressing an Insecticidal Double-Stranded RNA. *Transgenic Res.* 25, 1–17. doi:10.1007/s11248-015-9907-3

Capalbo, D. M. F., Macdonald, P., Fernandes, P. M. B., Rubinstein, C., and Vicién, C. (2020). Familiarity in the Context of Risk Assessment of Transgenic Crops: Focus on Some Countries in the Americas. *Front. Bioeng. Biotechnol.* 7, 463. doi:10.3389/fbioe.2019.00463

Garcia-Alonso, M., Hendley, P., Bigler, F., Mayeregger, E., Parker, R., Rubinstein, C., et al. (2014). Transportability of Confined Field Trial Data for Environmental Risk Assessment of Genetically Engineered Plants: a Conceptual Framework. *Transgenic Res.* 23, 1025–1041. doi:10.1007/s11248-014-9785-0

Glenn, K. C., Alsop, B., Bell, E., Goley, M., Jenkinson, J., Liu, B., et al. (2017). Bringing New Plant Varieties to Market: Plant Breeding and Selection Practices Advance Beneficial Characteristics while Minimizing Unintended Changes. *Crop Sci.* 57, 2906–2921. doi:10.2135/cropsci2017.03.0199

Kramer, C., Brune, P., McDonald, J., Nesbitt, M., Sauve, A., and Storck-Weyhermueller, S. (2016). Evolution of Risk Assessment Strategies for Food and Feed Uses of Stacked GM Events. *Plant Biotechnol. J.* 14, 1899–1913. doi:10.1111/pbi.12551

Ladics, G. S., Bartholomaeus, A., Bregitzer, P., Doerrer, N. G., Gray, A., Holzhauser, T., et al. (2015). Genetic Basis and Detection of Unintended Effects in Genetically Modified Crop Plants. *Transgenic Res.* 24, 587–603. doi:10.1007/s11248-015-9867-7

Nakai, S., Hoshikawa, K., Shimono, A., and Ohsawa, R. (2015). Transportability of Confined Field Trial Data from Cultivation to Import Countries for Environmental Risk Assessment of Genetically Modified Crops. *Transgenic Res.* 24, 929–944. doi:10.1007/s11248-015-9892-6

Pilacinski, W., Crawford, A., Downey, R., Harvey, B., Huber, S., Hunst, P., et al. (2011). Plants with Genetically Modified Events Combined by Conventional Breeding: An Assessment of the Need for Additional Regulatory Data. *Food Chem. Toxicol.* 49, 1–7. doi:10.1016/j.fct.2010.11.004

Privalle, L. S., Chen, J., Clapper, G., Hunst, P., Spiegelhalter, F., and Zhong, C. X. (2012). Development of an Agricultural Biotechnology Crop Product: Testing from Discovery to Commercialization. *J. Agric. Food Chem.* 60, 10179–10187. doi:10.1021/jf302706e

Que, Q., Chilton, M.-D. M., de Fontes, C. M., He, C., Nuccio, M., Zhu, T., et al. (2010). Trait Stacking in Transgenic Crops: Challenges and Opportunities. *gmcrops* 1 (4), 220–229. doi:10.4161/gmcr.1.4.13439

Schnell, J., Steele, M., Bean, J., Neuspiel, M., Girard, C., Dormann, N., et al. (2015). A Comparative Analysis of Insertional Effects in Genetically Engineered Plants: Considerations for Pre-market Assessments. *Transgenic Res.* 24, 1–17. doi:10.1007/s11248-014-9843-7

Signorini, A. M., Abratti, G., Grimi, D., Machado, M., Bunge, F. F., Parody, B., et al. (2018). Management of Field-Evolved Resistance to Bt Maize in Argentina: A Multi-Institutional Approach. *Front. Bioeng. Biotechnol.* 6, 67. doi:10.3389/fbioe.2018.00067

Vesprini, F., Maggi, A. I., López Olaciregui, M., and Módena, N. A. (2020). Transportability of Conclusions from Confined Field Trials: A Case Study Using the Virus Resistant Transgenic Bean Developed in Brazil. *Front. Bioeng. Biotechnol.* 8, 815. doi:10.3389/fbioe.2020.00815

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Bioengineering and Biotechnology

OPINION
published: 04 February 2022
doi: 10.3389/fbioe.2022.837635

Regional Initiatives in the Western Hemisphere as a Contribution to the Safe Biotechnology Development

Pedro J. Rocha-Salavarrieta *

Inter-American Institute for Cooperation on Agriculture (IICA), San José, Costa Rica

Keywords: biosafety, biotechnology regulation, institutionalism, GMOs, gene editing, regulatory cooperation

INTRODUCTION

Under the spirit of collaboration and coordination, countries have created several instruments to address biotechnology and biosafety (B&B) issues. For instance, the CBD (United Nations, 1992), the CPB (Secretariat of the Convention on Biological Diversity, 2000), and the Codex Alimentarius guidelines on risk assessment (FAO, 2021). In addition, neighboring countries have reached some agreements to consider B&B issues from a regional perspective.

Whether global or regional, such instruments establish general guidelines that seek similarity in the treatment of certain issues or the application of specific requirements. For example, not to affect transboundary movement or trade, taking advantage of technological developments, assessing risks in an objective manner, promoting food safety and quality, and achieving global environmental sustainability, thus favoring comprehensive and safe development.

GROUP 5 OF THE AGRICULTURAL COUNCIL OF THE SOUTH (G5-CAS)

The Agricultural Council of the South (CAS, for its name in Spanish), created in April 2003, is integrated by the ministers of agriculture of Argentina, Bolivia, Brazil, Chile, Paraguay, and Uruguay. It is a forum for consultation and coordination of regional actions, whose purpose is to define the priorities of the agricultural agenda and take positions on issues of regional interest in order to coordinate specific actions (CAS, 2021). The CAS hold regular meetings and presents very concise and pragmatic "Ministerial Declarations".

To identify short- and medium-term joint actions for regional cooperation, the CAS has the Agricultural Policy Coordination Network (REDPA) that embraces the Directors of Agricultural Policies and its various Technical Groups, including Technical Group 5 (G5-CAS) on Public Policies on Biotechnology. The G5-CAS includes national experts from five of the six CAS countries (except Bolivia) who analyze different topics, then generate regional position proposals on strategic B&B issues, according to the needs of the region, for Council's ministers debate and approval.

G5-CAS recognized the importance of genome editing (GnEd) for agriculture development, the need for having science-based decisions to promote research and development, and to avoid non-justified barriers to international trade. Based on that, the ministers agreed on fostering the technology; calling in different international fora for the application of transparent science-based regulatory frameworks; to promote capacity building activities; and to encourage the collaborative work for exchanging information about products development and regulatory advances (**Table 1**). This clear institutional support to the technology explains, in part, the technical and regulatory advances on GnEd in the Southern Cone and its encouragement to other countries and regions.

OPEN ACCESS

Edited by:
Carmen Vicien,
University of Buenos Aires, Argentina

Reviewed by:
Karim Maredia,
Michigan State University, United States
Clara Rubinstein,
Bayer, Argentina

***Correspondence:**
Pedro J. Rocha-Salavarrieta
Pedro.Rocha@iica.int

Specialty section:
This article was submitted to Biosafety and Biosecurity, a section of the journal Frontiers in Bioengineering and Biotechnology

Received: *16 December 2021*
Accepted: *17 January 2022*
Published: *04 February 2022*

Citation:
Rocha-Salavarrieta PJ (2022) Regional Initiatives in the Western Hemisphere as a Contribution to the Safe Biotechnology Development. Front. Bioeng. Biotechnol. 10:837635. doi: 10.3389/fbioe.2022.837635

TABLE 1 | Some statements issued by G5-CAS.

Ministerial declaration (date)	Statement
XXXVII-2019 (28–29/05/2019)	Statement III. Low level presence of genetically modified organisms not authorized by the importing country. (LLP) http://consejocas.org/wp-content/uploads/2019/05/XXXVII-RO-CAS-Declaraci%C3%B3n-III.-Low-Level-Presence.pdf
XXXVI-2018 (20–21/09/2018)	Statement I. Access to third markets for GMO products and their derivatives http://consejocas.org/wp-content/uploads/2018/09/XXXVI-RO-CAS-Declaraci%C3%B3n-I.-Acceso-a-terceros-mercados-de-productos-OGM-y-sus-derivados.pdf Statement II. Genome Editing Techniques http://consejocas.org/wp-content/uploads/2018/09/XXXVI-RO-CAS-Declaraci%C3%B3n-II.-T%C3%A9cnicas-de-Edici%C3%B3n-G%C3%A9nica.pdf
XXXV-2018 (3–4/05/2018)	Statement I. Priorities of the Agricultural Council of the South Opening to third markets of biotechnology events in the region, such as GMOs and NTBs http://consejocas.org/wp-content/uploads/2018/05/Declaraci%C3%B3n-I.pdf
XXXIV-2017 (27/08/2017)	Statement III. New breeding techniques and access of GM products to third markets http://consejocas.org/wp-content/uploads/2017/08/Declaracio%CC%81n-III-Nuevas-tecnologi%CC%81as-de-mejoramiento-y-acceso-de-productos-GMs-a-terceros-mercados-1.pdf
XXXII-2016 (3–4/11/2016)	Statement III. Negotiation of the Cartagena Protocol (COP-MOP8) http://consejocas.org/wp-content/uploads/2016/11/Declaracio%CC%81n-III-Negociacio%CC%81n-del-Protocolo-de-Cartagena.pdf Statement IV. Development of new breeding technologies http://consejocas.org/wp-content/uploads/2016/11/Declaracio%CC%81n-IV-Desarrollo-de-Nuevas-Tecnologi%CC%81as-de-Mejoramiento.pdf

NORTH AMERICAN BIOTECHNOLOGY INITIATIVE (NABI)

The NABI, signed in October 2003, was a high-level policy dialogue on topics related to agricultural biotechnology for regulators from Canada, Mexico, and United States. Its objectives were to exchange information among members, discuss common interest topics, and promote the development of innovative and cooperative approaches in order to regulate products of agricultural biotechnology as well as identify areas for further cooperation ranging from scientific research, collaborations, market access, and regulatory regimes.

Remarkably, NABI reached the trilateral arrangement on the "Documentation Requirements for Living Modified Organisms for Food or Feed, or for Processing (LMO/FFP's)", an important mechanism that allowed the implementation of Article 18.2 (a) of the CPB. Apart from facilitating Mexico to accomplish its obligations to the CPB without disrupting intra-regional trade, this arrangement ensured certainty in the trading environment between parties and non-parties of CPB (Winkles, 2004), which has been demonstrated, as global trade of LMOs continues today based on this arrangement.

INITIATIVE FOR CENTRAL AMERICA IN BIOTECHNOLOGY AND BIOSAFETY

The ICABB, created in March 2013, is a platform for dialogue and technical exchange on issues of interest in agricultural B&B (IICA, 2013). It comprises the coordinators of the National Technical Commissions of Biosafety of Belize, Costa Rica, El Salvador, Guatemala, Honduras, Nicaragua, Panama, and the Dominican Republic.

Operationally, ICABB organizes meetings to present regulatory advances, propose training, establish communication activities, and analyze documents in order to fulfill its consultative function on biosafety issues for the countries of the region. Some ICABB achievements include a workshop on risk assessment (IICA/UNEP-GEF, 2013) and the review of a technical document -proposed by one of its members- that indirectly contributed to both the generation of a national biosafety regulation and the support to the customs union agreement explained further down.

CUSTOMS UNION AGREEMENT EL SALVADOR-GUATEMALA-HONDURAS

The customs union agreement between Guatemala and Honduras is a form of trade integration, operating since June 2017 (SIECA/CEIE, 2018), and expanded with El Salvador in August 2018 (SICA, 2021). This instrument is a deep integration process, led by a ministerial committee of the three member States that promotes free transit of goods and services. Among many other issues, the ministerial committee has discussed and taken decisions on the use of B&B for the agricultural sector.

The tri-national group proposed the "Technical Rule on Biosafety of Living Modified Organisms for Agricultural Use, RT65.06.01:18″ through a strict process of technical discussions and formal protocols (for regular meetings; participation of different agencies-agriculture, environment, and economics-; and public consultations, both national and international).

Interestingly, although this rule is a multinational instrument, ratified and implemented by each country, it has not displaced national legislations, but on the contrary, it complemented them by providing technical and legal support for the revision (Honduras) and generation (Guatemala) of their biosafety regulatory frameworks, offering greater technical, operational, and administrative clarity (SIECA, 2019).

Consequently, Honduras generated and approved the authorization procedure for applications related to the use of precision biotechnology (The Gazette Official Journal of Honduras, 2019). Guatemala created its Technical Committee of Agricultural Biotechnology (named CTBAG; Central American Journal of Guatemala, 2019), and established its biosafety legal framework of LMOs that includes specific provisions addressing the regulatory status of GnEd products (Central American Journal of Guatemala, 2019a). Due to its subsequent integration, El Salvador advances in the discussion and the eventual issuance of a biotech regulation for agriculture.

Therefore, through transparent, predictable, and rigorous regulatory B&B national systems, the customs union agreement strengths the agricultural sector, reinforces the national institutionality, provides new opportunities for developers, and offers farmers access to biotechnological alternatives.

DISCUSSION

In a global scenario characterized by complex commercial, social, political, legal, technological, productive, and environmental dynamics, the relationship and negotiation between countries are imperative, and lead to the promotion of multilateral cooperation. Due to heterogeneity among countries, complete harmonization of laws or standards related to LMOs will probably not be possible, but regulatory cooperation is an effective alternative in that direction.

Regulatory cooperation through regional initiatives/platforms in B&B helps to optimize the technical resources available in the countries and regions, allowing to identify potential conflicts and, more importantly, to determine possible ways to resolve them [e.g. NABI and Art.18.22 (a) of CPB]. In addition, their agile and informal governmental schemes (characterized by administrative flexibility and technical rigor) contribute to the optimization of decision-making. Regional initiatives provide more clarity, transparency, and confidence in the assessment systems and institutions, opening the door to the use of common (and simplified) procedures based on the recognition of third countries assessments when justified (e.g. Paraguay) as well as considering the evaluations and regulatory decisions of peers in other countries. With that, work duplications avoid, resources optimize, and response times accelerate, which is relevant for international trade.

The nonbinding decisions taken in regional initiatives offers important technical orientations and policy references for national regulations, which have caused positive impact on biotechnology access, technology transfer, and product commercialization. In addition, such platforms encourage and guide other countries and regions. For instance, CAS and NABI stimulated ICABB creation, and the later contributed some elements for the technical rule of the Customs Union Agreement. In this manner, regional initiatives show both possible options and practical pathways for addressing current and future biosafety issues in a very articulate way.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

ACKNOWLEDGMENTS

The PR-S acknowledges Alejandra Sarquis (CAC Secretariat) for providing information about CAC and Lloyd Day (Deputy Director of IICA) for his valuable comments on this manuscript.

REFERENCES

CAS (Consejo Agropecuario del Sur) (2021). Consejo Agropecuario del Sur. Available at: http://consejocas.org/consejo-agropecuario-del-sur/(Accessed December 15, 2021).

Central American Journal of Guatemala (2019). Acuerdo Ministerial No. 270-2019 acuérdese Crear el Comité Técnico de Bioseguridad Agrícola (CTBAG). Available at: https://visar.maga.gob.gt/visar/2019/20/AM270-2019.pdf (Accessed January 9, 2022).

Central American Journal of Guatemala (2019a). Acuerdo Ministerial No. 281-2019 acuérdese Aprobar el "Manual de procedimientos técnicos para uso confinado, experimental, pre-comercial y comercial de semilla genéticamente modificada derivado de la implementación del reglamento técnico de bioseguridad 65.06.01:18". Available at: https://visar.maga.gob.gt/visar/2019/20/AM271-2019.pdf (Accessed January 9, 2022).

FAO (Food and Agriculture Organization from the United Nations) (2021). The Role of Codex in Biotechnology. Available at: https://www.fao.org/fao-who-codexalimentarius/thematic-areas/biotechnology/en/(Accessed December 15, 2021).

IICA/UNEP-GEF (2013). "Inter-American Institute for Cooperation on Agriculture, UNEP-GEF Project: Implementation of a National Biosafety Framework for Costa Rica," in *Proceedings of the Workshop on Risk Assessment in Biosafety (San Jose, February 26th February to March 2nd, 2013)* (San Jose, Costa Rica: IICA). Available at: http://repiica.iica.int/docs/b3116i/b3116i.pdf (Accessed December 15, 2021).

IICA (Inter-American Institute for Cooperation on Agriculture) (2013). *Initiative for Central America on Biotechnology and Biosafety: Towards the Development of a Regional Mechanism*. San José, Costa Rica: IICA. Available at: https://www.researchgate.net/publication/255950341_Initiative_for_Central_America_on_Biotechnology_and_Biosafety_ICABB (Accessed December 15, 2021).

Secretariat of the Convention on Biological Diversity (2000). *Cartagena Protocol on Biosafety to the Convention on Biological Diversity*. Montreal: text and annexes. Available at: https://www.cbd.int/doc/legal/cartagena-protocol-en.pdf (Accessed December 15, 2021).

SICA (Sistema de Integración Centroamericana) (2021). Unión Aduanera. Available at: https://www.sica.int/iniciativas/aduanas (Accessed December 15, 2021).

SIECA/CEIE (Secretaria de Integración Económica Centroamericana/Centro de Estudios para la Integración Económica) (2019). Libro Blanco de la Unión Aduanera Guatemala Honduras. Available at: http://www.oie.sieca.int/documentos/ver/Libro%20Blanco%20Union%20Aduanera%202018.10.09.pdf (Accessed December 15, 2021).

SIECA (Secretaria de Integración Económica Centroamericana) (2018). Reglamento Técnico de Bioseguridad de Organismos Vivos Modificados para Uso Agropecuario. RT 65.06.01:18 Available at: https://www.sieca.int/index.php/download/resolucion-no-60-2019-aprueba-rt-65-06-0118-bioseguridad-de-organismos-vivos-para-uso-agropecuario/(Accessed December 15, 2021).

The Gazette Official Journal of Honduras (2019). Acuerdo N° 8-SENASA-2019-Procedimiento de autorización para solicitudes relacionadas con el uso de nuevas

técnicas de mejoramiento genético (biotecnología de precisión). Available at: http://extwprlegs1.fao.org/docs/pdf/hon190734.pdf (Accessed January 9, 2022).
United Nations (1992). Convention on Biological Diversity. Available at: https://www.cbd.int/doc/legal/cbd-en.pdf (Accessed December 15, 2021).
Winkles, D. (2004). "Statement of David Winkles," in *Committee on Agriculture House of Representatives* (Washington: Review of Agricultural Biotechnology). Serial No. 108-34 Available at: https://books.google.co.cr/books?id=gSQsdZx0akAC&printsec=frontcover&hl=es&source=gbs_ge_summary_r&cad=0#v=onepage&q=trilateral&f=false (Accessed December 15, 2021).

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Bioengineering and Biotechnology

BRIEF RESEARCH REPORT
published: 18 February 2022
doi: 10.3389/fbioe.2021.801891

Attitudes of the Ecuadorian University Community Toward Genetically Modified Organisms

Carlos Román Collazo[1]*, *Karen Chacha Guerrero*[1], *Tatiana Loja Mejia*[1], *Diego Andrade Campoverde*[1] and *Yenima Hernández Rodriguez*[2]

[1]Faculty of Biochemistry and Pharmacy, Catholic University of Cuenca, Cuenca, Ecuador, [2]Faculty of Clinical Psychology, Universidad Católica de Cuenca, Cuenca, Ecuador

Introduction: The acceptance of genetically modified organisms (GMOs) by the civilian population in Ecuador is a controversial issue, where beliefs and practices are determinant. In Ecuador, the use of GMOs for research or productive purposes has been banned since 2008; however, the current position of the population toward this technology is unknown.

Objective: The aim of the study was to explain the attitude toward GMOs in the Ecuadorian university population based on sociodemographic variables, knowledge, beliefs, practices, and bioethical approach.

Methods: A validated survey was applied to 719 students and teachers of the Catholic University of Cuenca through Google Forms. The collected data were processed using SPSS 23.0 software. Multivariate and linear regression analyses were used to explain the attitude toward GMOs based on the variables studied.

Results: Partial approval of GMO use is research-oriented, with a rejection toward food. The linear regression model explained 65% of the variance of attitude toward GMOs from the beliefs, practices, knowledge, and bioethical approach variables. The sociodemographic variables were completely excluded from the model due to the absence of statistical significance.

Conclusions: The incipient acceptance of GMOs in the academic sector corroborates a transformation in the thinking of Ecuadorian civil society. Considerations on the use of GMOs are supported by a bioethical approach that leans toward a pragmatic utilitarianism based on the immediate or mediate benefits of the technology.

Keywords: attitudes, genetically modified organisms, biotechnology, transgenic, genetic engineering

OPEN ACCESS

Edited by:
Carmen Vicien,
University of Buenos Aires, Argentina

Reviewed by:
Sandra Sharry,
National University of La Plata, Argentina
Danilo Fernández Rios,
Universidad Nacional de Asunción, Paraguay

***Correspondence:**
Carlos Román Collazo
cromanc@ucacue.edu.ec

Specialty section:
This article was submitted to Biosafety and Biosecurity, a section of the journal Frontiers in Bioengineering and Biotechnology

Received: *26 October 2021*
Accepted: *07 December 2021*
Published: *18 February 2022*

Citation:
Román Collazo C, Chacha Guerrero K, Loja Mejia T, Andrade Campoverde D and Hernández Rodriguez Y (2022) Attitudes of the Ecuadorian University Community Toward Genetically Modified Organisms. Front. Bioeng. Biotechnol. 9:801891. doi: 10.3389/fbioe.2021.801891

INTRODUCTION

The use of genetically modified organisms (GMOs) in research, health, and food is a reality since the end of the 20th century (Manoj and Ratwan, 2018) (Robinson AW and Rajakaruna, 2016). After almost 40 years of its establishment as a technology, its acceptance and use are spreading to countries in several continents, mainly America (Paull and Hennig, 2019).

Even when there is unobjectionable evidence of the socioeconomic advantages of GMOs (Smyth et al., 2015); some regions of the planet are reluctant to adopt them as part of technological or productive systems (Martin et al., 2017). In Latin America, countries such as Venezuela, Peru, and

Ecuador continue to object to the implementation of GMO technology under the bioethical principle of precaution, hindering its development at regional and local levels (Gatica-Arias, 2020).

In Ecuador, the constitution and other legal figures limit GMO research and production (Gudynas, 2017), even though consulting entities have suggested its application at the local level (Trigo et al., 2002). It is also paradoxical in this prohibitive context, the use of GMO products by the population in high diversity and magnitude (Galeas, Yépez, and Lascano, 2016).

In 2008, a massive consultation about GMOs was held within the framework of the new Ecuadorian constitution. At this time, Ecuador was considered free of "transgenic organisms" and "risky biotechnologies" (Bravo 2017). Although there are investigations on the acceptance of GMOs in the Ecuadorian population, they are restrained to food consumption. There is no reference to the current perception of the Ecuadorian population toward GMOs in research or pharmaceutical applications. It is also unknown what variables may be associated with the position of the Ecuadorian population toward GMOs. The objective of the research was to explain the attitudes toward GMOs in the Ecuadorian university population based on sociodemographic variables, beliefs, practices, and bioethical approach.

MATERIALS AND METHODS

Research Design

The research followed a non-experimental, observational, cross-sectional, and explanatory design. The study population was the university community of the Catholic University of Cuenca (UCACUE), Ecuador, during the period March–August 2020. The total population was 14,482 teachers and students. The sampling was non-probabilistic, reaching the total study population. The inclusion criterion was to be a member of UCACUE during the research period. The exclusion criterion was to perform service or administrative functions at UCACUE. The sample was 729 members of the educational community.

Survey

A survey on attitudes toward GMOs was elaborated according to Pardo and collaborators (Pardo, Midden, and Miller 2002) (**Annex 1**). The survey was applied online using the Google Forms platform, which was administered through the UCACUE email management system. The survey was available online from November 2019 to August 2020. The survey was previously validated by piloting with reliability by Cronbach's alpha (α = 0.81) and content validity by V Aiken (0.82–0.97). The dimension practices with GMOs (6 items; α = 0.721; V Aiken = 0.84–0.96), attitude toward GMOs (6 items; α = 0.862; V Aiken = 0.89–0.97), beliefs regarding GMOs (16 items; α = 0.883; V Aiken = 0.89–0.97), and the bioethical approach (5 items; α = 0.796; V Aiken = 0.81–0.94) were measured on a Likert scale (1, strongly disagree, to 5, strongly agree). In addition, GMO knowledge (9 items; α = 0.869; V Aiken_0.86–0.97) was assessed on a scale of 0–9. Sociodemographic variables such as age, sex, place of residence, religion, educational level, academic training, family economic income, self-perceived GMO knowledge, and food expenditures were explored. The survey was self-administered with an average response time of 12 min.

Statistical Processing

The data were stored in an electronic database and processed using IBM SPSS 23.0 statistical software. Frequency analysis, measures of central tendency and position (mean, confidence intervals, percentiles), and dispersion (standard deviation, range) were used. Differences between groups were established using the Wilcoxon test for comparison of means for different groups. The effect of the independent variables was done by multivariate regression analysis and subsequently by linear regression. The assumptions of the model such as the absence of collinearity were analyzed by graphs, the Durbin Watson coefficient, and correlation between the independent variables. The significance level of all tests was less than or equal to 0.050.

Ethical Aspects

The research complied with the ethics of research with human subjects using informed consent and the voluntary approval of the participants. Prior informed consent was obtained from all those involved. The research objectives were presented in writing, highlighting the importance of the research. Anonymity and the willingness of respondents and interviewees to disclose information were respected.

RESULTS

Sociodemographic Characteristics of the Sample

The average age of the sample was 36.92 +− 11.61 (95% CI, 36.07–37.76) years. There was similarity in the proportions of men (48.6%) and women (51.4%) (X^2 = 0.605; p = 0.437), with urban residence being the majority (83.7%) over rural residence (16.3%) (X^2 = 330.701; p = 0.000). The educational level of the majority was in the Master's–Doctorate category (51.3%), decreasing for higher basic level (28.0%) and university level (20.7%) (X^2 = 111.712; p = 0.000).

Figure 1 shows the relative frequency of the variables household income, food expenditures, self-perceived knowledge about GMOs, and academic training. The most frequent family income was higher than 1,600 USD (X^2 = 111.712; p = 0.000). Expenditures on food were centered between 200 and 400 USD (X^2 = 98.181; p = 0.000). The predominant self-perceived knowledge corresponded to the medium category (X^2 = 284.502; p = 0.000). The predominant training area was health sciences, followed by social sciences. The low level of training in bioethics of the population is highlighted (X^2 = 224.126; p = 0.000).

The majority of the sample is religiously Catholic (80.7%). The rest of the categories such as Protestant (4.9%), Muslim (0.5%), Afro-Ecuadorian (0.3%), and others (3.8%) showed lower frequencies (X^2 = 2,174.410; p = 0.000). A total of 9.7% did not adhere to any religion.

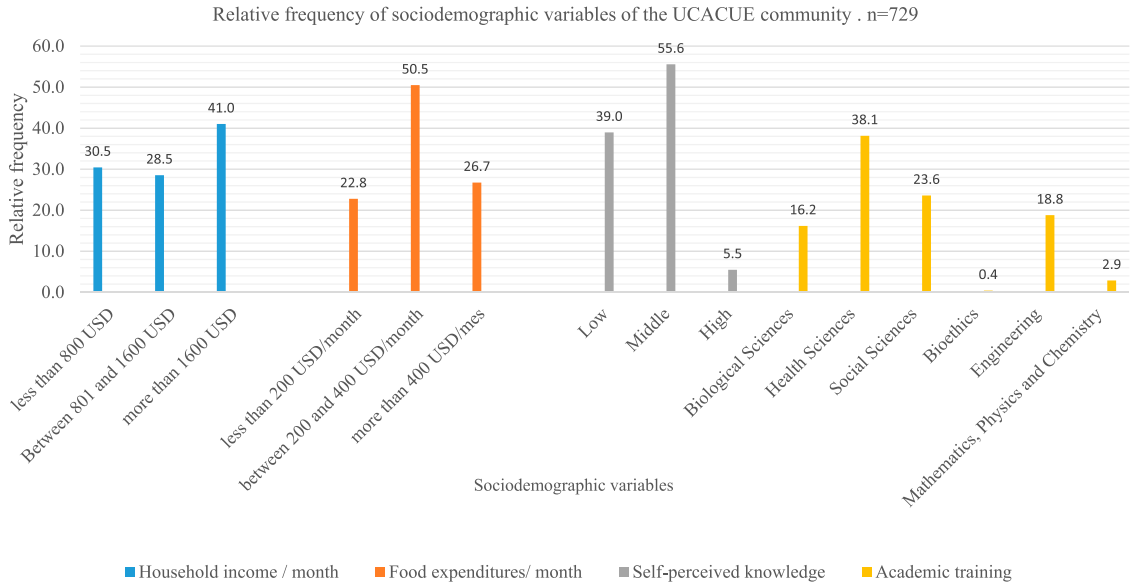


FIGURE 1 | Sociodemographic characteristics of the UCACUE community, March–August 2020, Ecuador.

TABLE 1 | Descriptive statistic of the attitude, beliefs, practices, and knowledge on GMO variables in UCACUE, 2020.

Variable	X +– SD	CI 95%	Min-Max	Instrument scale
Attitude toward GMO	3.03 +– 1.01	2.96–3.11	1–5	1–5
Beliefs about GMO	3.33 +– 0.70	3.28–3.38	1–5	1–5
Practices with GMO	2.54 +– 0.80	2.48–2.60	1–5	1–5
Knowledge about OGM	1.95 +– 1.66	1.81–2.05	0–8	0–9
Bioethical approach	2.98 +– 0.94	2.92–3.06	1–5	1–5

X +– SD-mean +– standard deviation.
CI, confidence interval.
Min, minimum.
Max, maximum.

TABLE 2 | Items that make up the attitude toward GMOs dimension.

Items that make up the attitude toward GMO dimension	Mean	Standard deviation	Mean's comparison (Wilcoxon's test)
A. I approve of the use of GMOs technology in the country under strict biosafety regulations	3.17	1.21	B > A
B. I am in favor of the use of GMOs in scientific research	3.39	1.20	Z = –5,638 p = 0.000
C. I approve GMOs for human consumption	2.72	1.17	A = F
D. I approve GMOs for feeding farmed animals	2.71	1.18	Z = –0.309 p = 0.758
E. I approve GMOs to produce medicines for humans and animals	3.06	1.18	A > C
F. I approve the use of GMOs for the care of the environment	3.16	1.26	Z = –10,822 p = 0.000
			C = D
			Z = –0.570 p = 0.568

Attitude Toward GMOs

Table 1 shows the descriptive statistics of the attitude, beliefs, practices, bioethics approach, and knowledge variables on GMOs. The average knowledge of the sample is located in the second quartile of the sampling distribution, suggesting a low level of familiarity with GMOs. Beliefs showed mean values close to the middle of the measurement scale. Practices showed a mean value below the middle of the scale, indicating less activity of individuals with GMOs in their daily lives. The bioethical approach also had a medium value, suggesting a deficit of bioethical thinking in the educational community. Finally, attitude toward GMOs, similar to the dimensions, showed a medium value in its behavior.

TABLE 3 | Summary measures of the linear regression model.[a,b]

R	R^2	Adjusted R^2	Standard error R2	R^2 change	Change statistics F change	df1	df2	Sig. F change	Durbin–Watson	ANOVA
0.807	0.652	0.647	0.600	0.652	149.444	10	719	0.000	1.951	F = 149.44 p = 0.000

Model	Unstandardized coefficients		Standardized coefficients	t	Sig	95.0% confidence interval B	
	B	Standard error	Beta			Lower limit	Upper limit
(Constant)	−0.697	0.165		−4.235	0.000	−1,021	−,374
Sex	0.078	0.046	0.039	1.709	0.088	−0.012	0.168
Place of residence	0.069	0.061	0.025	1.118	0.264	−,052	0.189
GMO beliefs	0.432	0.043	0.300	9.998	0.000	0.347	0.516
Practices with GMOs	0.147	0.033	0.117	4.507	0.000	0.083	0.211
GMO knowledge	0.050	0.014	0.082	3.460	0.001	0.021	0.078
Bioethical approach toward GMO	0.106	0.006	0.495	16.867	0.000	0.094	0.118
Religion	0.020	0.014	0.033	1.472	0.141	−0.007	0.048
Food expenditures (USD)	−0.047	0.038	−0.032	−1.217	0.224	−0.122	0.029
Household income (USD)	0.051	0.033	0.042	1.567	0.118	−0.013	0.115
Educational level	0.005	0.038	0.004	0.136	0.892	−0.069	0.080
Academic training	0.011	0.016	0.015	0.663	0.508	−0.21	0.42

[a]*Predictors: (constant), sex, place of residence, GMO beliefs, practices with GMOs, GMO knowledge, bioethical approach toward GMO, religion, food expenditures (USD), household income (USD), educational level, and academic training.*

[b]*Dependent variable: attitude toward GMOs.*

Table 2 shows the attitude toward GMOs according to the items that make up the dimension—the items with the lowest scores corresponded to the use of GMOs as food in humans or livestock. The highest acceptance corresponded to the use of GMOs as a means of research. There was a medium consensus on the use of GMOs for environmental protection under appropriate biosafety standards.

The modeling of attitude toward GMOs showed the properties of the model and the explanatory variables (**Table 3**). The proposed model was statistically significant in explaining the attitude toward GMOs of the UCACUE educational community. The variables adjusted to the model were beliefs, practices, knowledge, and bioethical approach toward GMOs by showing statistical significance. The variables entered showed a Durbin–Watson coefficient of 1.95, with tolerance and VIF adequate, ensuring the absence of collinearity and correlation between the explanatory variables. The rest of the variables was discarded because their inclusion in the model was not significant. The calculated coefficient of determination explains 65% of the attitude with a respective contribution of beliefs (43.4%), practices (14.7%), bioethical approach (10.5%), and knowledge (5%). Therefore, the modeling equation responds to the straight line shown:

$$\begin{aligned} Attitude\ towards\ GMO = & -0.674 + 0.434\,(Beliefs) \\ & + 0.147\,(Practices) \\ & + 0.050\,(Knowledge) \\ & + 0.105\,(Bioethical\ approach)\,. \end{aligned}$$

DISCUSSION

Public attitudes towards biotechnology have been explored since the emergence of GMOs. The introduction of GMOs into human life has been marked by a dichotomy: acceptance or rejection. Their varied applications lead to a different position depending on the usefulness of the GMO, revealing a pragmatic and utilitarian position in the popular reflection on the genetic event. The perception of risk/benefit determines rejection or acceptance (Dass, Anjum, and Gupta 2018). Differences in perceived risk between experts and civil society have been found to accentuate divergences in acceptance (Savadori et al., 2004).

Also, people's attitudes toward technology and its products are linked to the perception of right and wrong (Dass et al., 2018). For example, communities accept GMOs in the biopharmaceutical industry and research with minimal objections. Likewise, transgenic drugs such as insulin, GH, erythropoietin, and others are generally viewed as good and receive some community approval (Rzymski and Królczyk 2016).

Kazana et al., in a pioneering study on attitudes towards transgenic forest trees, demonstrated a similarity of attitudes among European and non-European university communities. The participants showed the criterion of using transgenic trees only in controlled areas without being released into the environment. The level of knowledge on transgenic trees leaned towards the concept but not towards the current status of their local or global use. There was also support for mandatory labeling as a requirement for free choice by the population. These considerations still suggest a state of

distrust towards this type of organism, which stigmatizes its presence in society (Kazana et al., 2015)

However, the perceived threat of GMOs and the perceived harm to health, the environment, or the natural order are criteria used by civil society (Scott et al., 2018). GMO foods are roundly rejected by fractions of civil society, perhaps as a manifestation of food phobia (Faccio and Nai, 2019). For example, in China, the population perception is primarily against GMO foods (Cui and Shoemaker, 2018), having political and economic causes related to structures that mediate the production process, marketing, and regulation. Similar situations are repeated in other countries such as Bosnia and Herzegovina (Bevanda et al., 2017), Tanzania (Mnaranara et al., 2017), and Mexico (Robayo et al., 2018).

In Ecuador, the attitude toward GMOs is marked by a set of social, educational, and political factors that have established a position of rejection (Paz-y-Mino et al., 2013). The conception of food sovereignty, biodiversity conservation, and environmental protection is part of the Ecuadorian cultural tradition, embodied at the constitutional level (Maluf et al., 2018). The view of GMOs as a threat to these cultural traditions is materialized in broad opposition in various political, academic, and civil circles (Bravo 2017).

Also the history of discredit in the mass media from a political perspective, the veto at the constitutional level for research and productive purposes, or the short term of its possible use under approval by presidential decree makes the population perceive threats over the benefits (Paz-y-Mino et al., 2013). Environmental campaigns propose a GMO-free Ecuador to achieve the health of the population and the conservation of the environment (Intriago and Bravo, 2015).

A relevant aspect to consider is the criterion for using GMOs under strict biosafety and biosecurity norms, revealing a reflection on the ethical assumptions that should guide the use of GMOs in Ecuador. Currently, there is no biosafety code for the use of GMOs in the country, even though its elaboration began in 2015 (Implementación del Marco Nacional de Bioseguridad, 2015).

According to the authors' criteria, several reasons may have an impact on the attitude toward GMOs. Some of them are associated with the massive lack of knowledge and the lack of instruction about new generation biotechnologies in curriculum at the higher primary and undergraduate levels. Research in students has found rational thinking in arguing the use of genetic technology, avoiding emotional arguments (Črne-Hladnik et al., 2012). That is why the authors support the approach used in research where students are the analytic unit and can be decisive to find ethical arguments in the population. It has also been corroborated that a lack of education and knowledge can be associated with the acceptance of GMOs (Cacciatore, 2021). According to the Dunning-Kruger model, the population's limited knowledge fosters high certainty of rejection towards GMOs. However, this fact is modified by the acquisition of more specific knowledge on the subject. There have also been minimal opportunities for debate between civil society and academic and political structures to educate and dialogue with the population about GMOs, resulting in a confrontation between science and the defenders of Pachamama (Mother Earth in Quechua language) (Paz-y-Mino et al., 2013).

Attitudes around GMOs have been associated with a set of sociodemographic variables such as religion, conceptions of life and nature, knowledge, educational level, area of academic training, geographic region, and culture (Öz et al., 2018). Modeling attitudes toward GM salmon in Malaysia using structural equations revealed the existence of a complex phenomenon with multiple explanatory variables (Amin et al., 2014). The predominant dimension was risk perception, although perceived benefits were also relevant. This fact coincides with findings in the Ecuadorian population (highly religious), where risks and benefits are perceived independently. The distorted beliefs of the Ecuadorian population on aspects related to GMOs such as biosafety and biosecurity have a direct impact on average acceptance. Also, the limited life experiences of the population with GMOs restrict practical knowledge.

The bioethical approach of the civilian population is a dimension explored in the explanation of attitudes towards GMOs. Exploring moral, utilitarian, personalist, and principal-based stances contribute to understanding the root causes of attitudes. Harfouche and collaborators showed that the ethical stance and values are decisive in the acceptance and trust of society towards GMOs as technology or their consumption as a product (Harfouche et al., 2021).

The collectivist and liberalist philosophical basis for using GMOs proposes two irreconcilable opposing extremes: greater good for the most significant number of people and individual freedom. Principlism endorses genetic modification as an ethical act proper to the autonomy of the scientist in the research. This position exalts freedom as the main good, ignoring possible consequences of scientific activity in the immediate future. The utilitarian approach arises the benefits obtained due to genetic modification of organisms for the people or where the benefit outweighs the existing risks (Appiah, 2015). This view argues for the extensive use of biotechnology to mitigate hunger in vast regions of the planet (Harfouche et al., 2021).

The anthropological personalist bioethical arguments propose humans as an end in themselves. The superiority of humans over the rest of the species justifies genetic modification, as long as the end itself is the wellbeing of humans. This anthropocentric position establishes the person over the rest of living organisms, minimizing the ecological conception of human life. However, there are more conciliatory positions with nature and living beings that integrate and respect living beings or the ecosystem as a whole. The biocentrist and ecocentrist currents have managed to reconcile humans with their environment to achieve the necessary sustainability of the ecosystem and curb environmental deterioration in this new era of the Anthropocene (Lee, 2017).

The virtue ethics proposes the acceptance of GMOs under strict *in situ* and *ex situ* regulatory measures. The application of bioethical principles such as responsibility and precaution allows for the regulation of GMO technology (Appiah, 2015). According to the author, the responsible use of GMO technology must fallows four main guidelines: search for the wellbeing of humans and their environment, future projection on possible effects, participation of all sectors of society in the approval of its use, and broad accessibility to all sectors of society.

Bremer et al., in their case study research on attitudes towards fast-growing transgenic salmon in Europe, emphasized a systemic and pluralistic reflection (Bremer et al., 2015). The participation of productive and scientific entities of private or public profile and civil society can bring together different ethical thoughts in an open dialogue between decision-makers and society. Furthermore, the use

oftools such as the ethics matrix for decision-making can facilitate divergent meeting thoughts. However, the authors consider the proposal made by Bremer et al. to be reductionist because it only includes the principlism bioethical approach and ignores the rest of the trends. Technology governance should consider diverse trends to receive the most significant acceptance and the least possible uncertainty.

What Approach Should be used in Ecuador for an Adequate Governance of GMOs?

Even though there is no research, communication, or educational strategies on implementing GMO technology at the national and local levels. The change of position of the Ecuadorian academic population will favor actions to develop genetic engineering and biotechnology. The results obtained show UCACUE as an agent of change in this process. Therefore, this focus group represents the future consumers, policy-makers, or developers of this organism. The development of seedbeds at UCACUE could be the strategy for developing GMOs at the local level. Through educational, communicational, and participatory strategies, teachers and students could reconcile scientific and ethical criteria about GMOs. This fact suggests that the academic community could manage the national level's research, implementation, and development of GMO technology.

The authors consider that the results obtained should be interpreted with caution due to the biases caused by the use of the questionnaire as a measurement instrument in a population of university students. Response biases related to the number of participants and cognitive biases such as the Dunning-Kruger effect may be present reducing the scope of the investigation.

GMOs have shown a clash of opinions between science and the passionate defense of national sovereignty, the environment, and human health in Ecuador. Passions have marginalized scientific thoughts for the sake of preserving national culture and identity, health, the environment, and Ecuador's good living. The effect has been to provoke attitudes of rejection and fear toward this technology from extreme positions. However, there is currently a slight change of position with a tendency toward acceptance in the academic sector, corroborating a transformation in the thinking of Ecuadorian civil society toward GMOs. Considerations on the use of GMOs are supported by an incipient bioethical stance that leans toward a pragmatic utilitarianism based on the immediate or mediate benefits of the technology.

The use of GMOs in Ecuador must contemplate a process of change in the civilian population's perception of them. Dialogue among the productive, technological, scientific, academic, civil sectors, and the minorities and indigenous communities of society will make it possible to unify criteria and smooth differences over in this field of technology. The intervention of variables such as knowledge, the bioethical approach, beliefs, and practices with GMOs would be decisive in achieving their inclusion within the Ecuadorian science and technology system under the perspective of responsible research and innovation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**; further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CR contributed to research design, survey design and validation, data processing, and statistical analysis; YH helped with survey design and validation, data processing, and statistical analysis; KC and TL assisted with data collection and database creation; and DA involved in research design. All authors participated in the drafting and final revision of the manuscript.

ACKNOWLEDGMENTS

The authors would like to thank Marcela Carrasco and Daniela Jaramillo for manuscript revision and also acknowledge UCACUE for institutional support for data collection and the respective authorizations for the research.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2021.801891/full#supplementary-material

REFERENCES

Amin, L., Azad, M. A. K., Gausmian, M. H., and Zulkifli, F. (2014). Determinants of Public Attitudes to Genetically Modified Salmon. *PLoS ONE.* 9 (1), e86174. doi:10.1371/journal.pone.0086174

Appiah, S. K. (2015). The Debate on the Use of Genetic Technology and Production of GM Foods in Ghana: Ethical Perspectives. *Cont. J. Afr. Stud.* 3 (2), 113–134. doi:10.4314/contjas.v3i2.5

Bevanda, L., Žilić, M., Ećimović, B., and Matković, V. (2017). Public Opinion toward GMOs and Biotechnology in Bosnia and Herzegovina In edited by A. Badnjevic, *CMBEBIH 2017 IFMBE Proceedings*. Singapore: Springer. doi:10.1007/978-981-10-4166-2_70

Bravo, E. (2017). Visiones y Tensiones Sobre el Debate de los Transgénicos en el Ecuador. *Perspectivas Rurales Nueva Época* 15 (30), 11–29. December. doi:10.15359/prne.15-30.1

Bremer, S., Millar, K., Wright, N., and Kaiser, M. (2015). Responsible Techno-Innovation in Aquaculture: Employing Ethical Engagement to Explore

Attitudes to GM Salmon in Northern Europe. *Aquaculture.* 437 (febrero), 370–381. doi:10.1016/j.aquaculture.2014.12.031

Cacciatore, M. A. (2021). Misinformation and Public Opinion of Science and Health: Approaches, Findings, and Future Directions. *Proc. Natl. Acad. Sci. USA*. 118 (15), e1912437117. doi:10.1073/pnas.1912437117

Črne-Hladnik, H., Hladnik, A., Javornik, B., Košmelj, K., and Peklaj, C. (2012). Is Judgement of Biotechnological Ethical Aspects Related to High School Students' Knowledge? *Int. J. Sci. Education.* 34 (8), 1277–1296. doi:10.1080/09500693.2011.572264

Cui, K., and Shoemaker, S. P. (2018). Public Perception of Genetically-Modified (GM) Food: A Nationwide Chinese Consumer Study. *Npj Sci. Food.* 2 (1), 10. doi:10.1038/s41538-018-0018-4

Dass, P., Anjum, N., and Gupta, D. (2018). Genetically Modified Organisms: Reliability Analysis and Perceptions. *J. Inf. Optimization Sci.* 39 (7), 1401–1415. doi:10.1080/02522667.2018.1441245

Faccio, E., and Guiotto Nai Fovino, L. (2019). Food Neophobia or Distrust of Novelties? Exploring Consumers' Attitudes toward GMOs, Insects and Cultured Meat. *Appl. Sci.* 9 (20), 4440. doi:10.3390/app9204440

Gatica-Arias, A. (2020). The Regulatory Current Status of Plant Breeding Technologies in Some Latin American and the Caribbean Countries. *Plant Cell Tiss Organ Cult.* 141 (2), 229–242. doi:10.1007/s11240-020-01799-1

Gbashi, S., Adebo, O., Adebiyi, J. A., Targuma, S., Tebele, S., Areo, O. M., et al. (2021). Food Safety, Food Security and Genetically Modified Organisms in Africa: a Current Perspective. *Biotechnol. Genet. Eng. Rev.* 37 (1), 30–63. doi:10.1080/02648725.2021.1940735

Gudynas, E. (2009). La Ecología Política del Giro Biocéntrico en la Nueva Constitución de Ecuador. *Rev.estud.soc.* 32 (June), 34–47. doi:10.7440/res32.2009.02

Harfouche, A. L., Petousi, V., Meilan, R., Sweet, J., Twardowski, T., and Altman, A. (2021). Promoting Ethically Responsible Use of Agricultural Biotechnology. *Trends Plant Sci.* 26 (6), 546–559. doi:10.1016/j.tplants.2020.12.015

Implementación del Marco Nacional de Bioseguridad (2015). Proyecto. Ecuador. Available at: http://www.ambiente.gob.ec/wp-content/uploads/downloads/2015/08/BIOSEGURIDAD.pdf.

Intriago Barreno, R. S., and Bravo Velásquez, E. (2015). Situación Actual del Ecuador Como Territorio Libre de Transgénicos. *Lv.* 18, 264–226. doi:10.17141/letrasverdes.18.2015.1606

Kazana, V. L., Tsourgiannis, V., Iakovoglou, C., Stamatiou, A., Alexandrov, S., and Araújo, S. (2015). Public Attitudes towards the Use of Transgenic Forest Trees: A Cross-Country Pilot Survey. *IForest - Biogeosciences and Forestry* 9 (2), 344. doi:10.3832/ifor1441-008

Lee, L. M. (2017). A Bridge Back to the Future: Public Health Ethics, Bioethics, and Environmental Ethics. *Am. J. Bioeth.* 17 (9), 5–12. doi:10.1080/15265161.2017.1353164

Maluf, F., Calaça, I., Freitas, P., and Augusto, S. (2018). La Naturaleza Como Sujeto de Derechos: Análisis Bioético de las Constituciones de Ecuador y Bolivia. *Rev. Latinoam. Bioet.* 18 (34–1), 155–171. doi:10.18359/rlbi.3030

Manoj, K., and Ratwan, P. (2018). Transgenic or Genetically Modified Farm Animals and Their Applications: a Review. *Res. Rev. J. Vet. Sci. Technology.* 5 (3), 25–34. doi:10.37591/rrjovst.v5i3.560

Martin, H. M., Durr, D., Smith, L. M., Finke, R., and Cherry, A. (2017). Analysis of GMO Food Products Companies: Financial Risks and Opportunities in the Global Agriculture Industry. *Afr. J. Econ. Sustainable Development.* 6 (1), 1–17. doi:10.1504/AJESD.2017.082813

Mnaranara, T. E., Zhang, J., and Wang, G. (2017). Public Perception Towards Genetically Modified Foods in Tanzania. *J. Anim. Plant Sci.* 27 (2), 589–602. Available at: http://www.thejaps.org.pk/docs/v-27-2/30.pdf.

Oleas, M., Tejada, E., and Lascano, R. (2016). Conocimientos y Aceptación de Alimentos Transgénicos en Adolescentes de la Provincia de Imbabura, Ecuador. *Rev. Esp Nutr. Comunitaria.* 26 (1), 9, 2016 . Available at: http://www.renc.es/imagenes/auxiliar/files/RENC_2016_1-02._Oleas_M_Conocimientos_transgenicos_Imbabura(1).pdf.

Öz, B., Unsal, F., and Movassaghi, H. (2018). Consumer Attitudes Toward Genetically Modified Food in the United States: Are Millennials Different? *J. Transnational Management.* 23 (1), 3–21. doi:10.1080/15475778.2017.1373316

Pardo, R., Midden, C., and Miller, J. D. (2002). Attitudes Toward Biotechnology in the European Union. *J. Biotechnol.* 98 (1), 9–24. doi:10.1016/s0168-1656(02)00082-2

Paull, J., and Hennig, B. (2019). *New World Map of Genetically Modified Organism (GMO) Agriculture: North and South America Acres Australia (January)* 101, 59–60. Available at: https://www.researchgate.net/profile/John-Paull/publication/336411623_New_World_Map_of_Genetically_Modified_Organism_GMO_Agriculture_North_and_South_America_85/links/5da01b7ba6fdcc8fc346da67/New-World-Map-of-Genetically-Modified-Organism-GMO-Agriculture-North-and-South-America-85.pdf.

Paz-y-Mino, C., Binsfeld, P., Torres, M., Arahana, V., Bravo, E., Fornasini, M., et al. (2013). *Transgénicos: Una Cuestión Científica*. 1ra ed. Quito. UDLA. Available at: https://www.researchgate.net/profile/Cesar-Paz-Y-Mino/publication/326504306_Transgenicos_Una_cuestion_cientifica/links/5b8817f3a6fdcc5f8b71fde6/Transgenicos-Una-cuestion-cientifica.pdf.

Robayo, A., Galindo, M., Yáñez, L., and Aldama, C. (2018). Measurement of Public Perception of GMOs With a Likert-type Scale. *Agrociencia (Montecillo).* 52 (5), 767–781. Available at: https://www.cabdirect.org/cabdirect/abstract/20203084608.

Robinson AW, T., and Rajakaruna, A. (2016). Application of Recombinant DNA Technology (Genetically Modified Organisms) to the Advancement of Agriculture, Medicine, Bioremediation and Biotechnology Industries. *J. Appl. Biotechnol. Bioeng.* 1 (3), 78. doi:10.15406/jabb.2016.01.00013

Rzymski, P., and Królczyk, A. (2016). Attitudes Toward Genetically Modified Organisms in Poland: To GMO or Not to GMO? *Food Sec.* 8 (3), 689–697. doi:10.1007/s12571-016-0572-z

Savadori, L., Savio, S., Nicotra, E., Rumiati, R., Finucane, M., and Slovic, P. (2004). Expert and Public Perception of Risk From Biotechnology. *Risk Anal.* 24 (5), 1289–1299. doi:10.1111/j.0272-4332.2004.00526.x

Scott, S. E., Inbar, Y., Wirz, C. D., Brossard, D., and Rozin, P. (2018). An Overview of Attitudes Toward Genetically Engineered Food. *Annu. Rev. Nutr.* 38 (1), 459–479. doi:10.1146/annurev-nutr-071715-051223

Smyth, S. J., Kerr, W. A., and Phillips, P. W. B. (2015). Global Economic, Environmental and Health Benefits from GM Crop Adoption. *Glob. Food Security.* 7 (diciembre), 24–29. doi:10.1016/j.gfs.2015.10.002

Trigo, E., Traxler, G., Pray, C., and Echeverría, R. (2002). *Biotecnología agrícola y desarrollo rural en América Latina y el Caribe. RUR-107. Serie de informes técnicos del Departamento de Desarrollo Sostenible.* Whashington DC: Banco Interamericano de Desarrollo. Available at: https://d1wqtxts1xzle7.cloudfront.net/47904405/getdocument-with-cover-page-v2.pdf?Expires=1630594858&Signature=OEyXxug6YrbEvMhzolqyEs3bm5e~flAZQ9QGRuk6vP2qwpGQlQHEKtbIKNQthhvEe1gmNKlJXtLZEWjkXlg7~MlzVguhPP78EJhtJkkKzLe3o1oiZ6gm0TbKRJGe76rscg1Xmk-F0f2fqcrlKFA~PhBecneUsGqInsjTxZsZfhd~xkDYlJh-TrZGtvAZmPGOeN5JzusVrsiEav8vU4-w2svZKvLZNGB8G0pFJzIWQ7L1ODSuVgUZxrRS2DzOWrm3hPRHzM2eyC1krI3e1u2a4nfWwQSLZ4gHQ8gxOXdK~z9PAro5kLbXfOZL-mZWGvIFxKpDqdmMbmCs3Oz3fZlUyw__&Key-Pair-Id=APKAJLOHF5GGSLRBV4ZA.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors, and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Bioengineering and Biotechnology

PERSPECTIVE
published: 21 February 2022
doi: 10.3389/fbioe.2022.835378

Genomic Editing: The Evolution in Regulatory Management Accompanying Scientific Progress

María Florencia Goberna, Agustina Inés Whelan, Perla Godoy and Dalia Marcela Lewi*

National Directorate of Bioeconomy, Secretariat of Food, Bioeconomy and Regional Development, MAGyP, Buenos Aires, Argentina

Argentina currently has a regulation for genome-editing products whose criteria were updated as consultations were received to determine the regulatory status of these products. The aim of this regulation is to consider all organisms (animals, micro-organisms and plants) under the same NBT resolution independently and without being linked to commercial Genetically Modified Organism (GMO) regulations. This gives certainty to local researchers and developers (teams of local developers and researchers), which can be seen in the number of developments and consultations carried out. It should be noted that early results showed that the speed of innovation of these technologies was increasing in a short time, giving more opportunity to local developers who showed interest in generating products in different species, crops and phenotypes.

Keywords: genome editing, innovation, regulation, new breeding techniques, modern biotechnology

OPEN ACCESS

Edited by:
Carmen Vicien,
University of Buenos Aires, Argentina

Reviewed by:
Stuart Smyth,
University of Saskatchewan, Canada
Heidi Mitchell,
Australian Government, Australia

****Correspondence:***
María Florencia Goberna
mgoberna@magyp.gob.ar

Specialty section:
This article was submitted to Biosafety and Biosecurity, a section of the journal Frontiers in Bioengineering and Biotechnology

Received: *14 December 2021*
Accepted: *27 January 2022*
Published: *21 February 2022*

Citation:
Goberna MF, Whelan AI, Godoy P and Lewi DM (2022) Genomic Editing: The Evolution in Regulatory Management Accompanying Scientific Progress. Front. Bioeng. Biotechnol. 10:835378. doi: 10.3389/fbioe.2022.835378

INTRODUCTION

To begin with, modern biotechnology can be defined as the technological application of genetic engineering tools for the improvement of crops, micro-organisms, and animals of agricultural interest, with the aim of generating benefits for farmers, consumers, industry, human and animal health, and the environment. That is why some of the purposes of this technology are to improve and increase agricultural production, reduce production costs, make more efficient use of resources, promote resilience to climate change while preserving the productive environment, and increase food safety, security and quality (Petracca et al., 2016; Duensing et al., 2018; Gavin et al., 2018; Eriksson et al., 2019).

Argentina, in particular, has increased its production of genetically modified (GM) crops, currently being the world's third largest producer of biotech crops, after the United States and Brazil with a degree of adoption of transgenic varieties that, in the case of soybean and cotton, represents 99% of total trade with these crops and 98% in the case of maize, demonstrating the high degree of acceptance and adoption of these technologies by Argentine farmers (ISAAA, 2018).[1]

Argentina is one of the first countries to have developed and applied modern biotechnology techniques since the late 1980s (Burachik and Traynor, 2002) being this the basis for the development of a sound regulatory framework, which was set in motion with the creation of the National Advisory Commission on Agricultural Biotechnology (CONABIA) in 1991 which, as an evaluation and

[1]https://argenbio.org/recursos/66-estadisticas-isaaa/128-evolucion-superficie-gm.

consultation body, has a multidisciplinary approach and is composed of experts representing different sectors such as environment, health and agriculture, which is why it is one of the first countries to have developed and applied modern biotechnology techniques (Burachik and Traynor, 2002).

With 30 years of experience in the regulation of products obtained through genetic engineering, Argentina has consolidated its experience and capacity to determine criteria for the biosafety analysis of these products, which are used in the production of pharmaceuticals and in the human and animal food industry.

It is through CONABIA that Argentina provides advice and training, and collaborates with other countries on biotechnology regulatory approaches and frameworks. It is worth noting that due to its trajectory, in 2014 CONABIA was recognised by FAO (Food and Agriculture Organization of the United Nations) as a "Reference Centre" for the Biosafety of Genetically Modified Organisms (GMOs) and its designation was renewed in 2019.

Genomic editing is part of the group of so-called "New Breeding Techniques" (NBT). Like transgenesis, genome editing is a genetic engineering tool whose application allows for more sustainable food production, more nutritious products and better protection of crops against pests, diseases and climatic adversities. The difference between NBTs and GMOs is that these innovative tools allow targeted and precise DNA modifications (Barrangou and Doudna, 2016; Knott and Doudna, 2018; Chen et al., 2019).

Argentina has carried out an update and improvement of its entire regulatory framework for both GMOs and NBTs in 2020. The characteristics of New Breeding Techniques (NBTs) regulatory measures require a prior scientific analysis, on a case-by-case basis, of organisms already obtained or to be obtained, in order to determine whether they fall within the scope of the regulations applicable to Genetically Modified Organisms (GMOs) or not. In other words, the regulatory framework for NBTs establishes the procedures to determine whether or not any organism obtained through new breeding techniques using modern biotechnology is covered by GMO regulations.

Current Official Regulation

In 2013, Argentina carried out a preliminary analysis on the state of the art of NBTs in the world. Two years later, Argentina officially published the first NBT regulation only for plants (Whelan and Lema, 2015). A few years later, in 2019, the NBT regulation for animals and microorganisms was published and the regulation for plants was updated (Whelan and Lema, 2019).

As mentioned above, during 2020 the NBT regulations was updated and simplified and was officially published in the following year under Resolution N° 21/2021.[2] This resolution is based on N° 763 of 17 August 2011 (Ministry of Agriculture, Livestock, and Fisheries) which uses the Cartagena Protocol.[3]

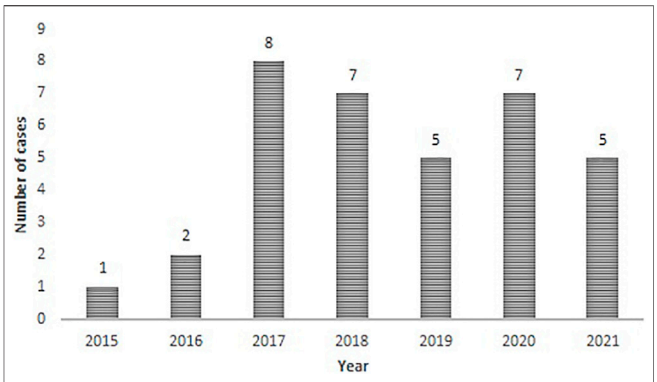


FIGURE 1 | Number of PCI cases analyzed between 2015 and 2021.

definition of GMO, understood as any living organism that possesses a new combination of genetic material obtained through the application of modern biotechnology. In addition, this new resolution includes the definition of "novel combination of genetic material" which refers to any change produced in the genome of the organism by the incorporation, in a stable and cohesive manner, of one or more genes or nucleic acid sequences that are part of a defined genetic construct.

This new NBT Resolution N° 21/2021 takes into account a procedure to determine whether a product obtained by NBT could be covered by the GMO regulation or not. This analysis begins when the interested party completes the Prior Consultation Instance (PCI) form according to the organisms of interest (plant, animal, or micro-organism). It should be clarified that this form can be submitted when the product is finished or when it is in the design stage). In case a PCI has been submitted for a product in the design stage, the developer must submit a second form when the product is finished, in order to verify whether the modifications made are the same as those described in the first PCI.

In this way, the National Directorate of Bioeconomy scientific-technical evaluation team and CONABIA analyze whether the product does not have a new combination of genetic material based on the information submitted. If there is indeed no new combination of genetic material, the product is non-GM and is considered as a conventional product. On the other hand, if the product has a new combination of genetic material, it is considered transgenic and must comply with GMO regulations according to the organism, animal, micro-organism and plant.

The analysis is carried out on a case-by-case basis, it is not limited to a specific list of techniques and allows for consultation when the product is at the design stage. Finally, the Commission must provide a response to the interested party within 80 working days. This updated regulation has specific annexes for animals, microorganisms and plants, as a guide to the information that the developer has to take into account when completing the PCI.

[2]https://www.boletinoficial.gob.ar/detalleAviso/primera/240529/20210208.
[3]https://www.cbd.int/doc/legal/cartagena-protocol-en.pdf.

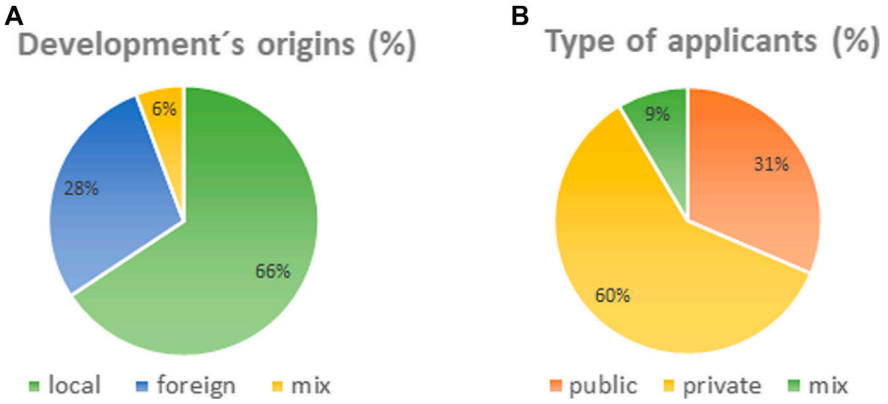


FIGURE 2 | (A) Origin of PCI submitted to the Coordination of Innovation and Biotechnology of the National Directorate of Bioeconomy. **(B)** Type of applicants that submitted PCIs.

PCIs Assessment

From the beginning of the application of the above-mentioned criteria, included in the first version of the NBT regulation, it was observed that these measures promoted the submission for consultation of developers (Whelan et al., 2020).

The analysis of the entire experience generated by the application of these regulations reveals the following conclusions about the PCI cases analyzed: 1) the developers can predict costs and period of time in the product development, even at the design stage; 2) the developers can put their products into the market sooner; 3) there are a greater phenotype varieties in different crops, animals and microorganisms; and 4) the speed of innovation of products obtained by NBTs is greater in relation to GMOs the innovation speed.

Additionally, among the cases analyzed, the following results were obtained: between 2015 and 2021 there were 35 PCI cases (**Figure 1**). The proportion of techniques used was 86% gene editing and 14% others NBT. The queries were made by 66% local developers; 28% foreign developers. Of that percentage, most of the foreign developments presented come from North America and the minority from Europe. Finally, 6% foreign developments were submitted by local companies. Regarding the type of applicants submitting PCIs, 60% were private companies, 31% public institutions and 9% were mixed entities (**Figure 2**). In contrast to the scenario of Argentina's development of GMO, the origin of developments and type of applicant, 95% are from foreign origin and 5% from national origin. Regarding NBTs, PCI submitted distribution by type of organism was: 57% crops, 29% animals, and 14% microorganisms. Talking about the state of the developments, 60% of the PCIs were hypothetical products, while the 40% of the PCIs were about real products. As an example of a local product developed in Argentina applying genetic edition, it could be mentioned the reduced enzymatic browning in potato tubers, obtained by a public research institution (González et al., 2020).

Innovation

In addition to the improvement of the regulations, which came into force in January 2021, and taking into account the analysis of Lewi and Vicién, (2020), other actions were launched to promote the approach of local developers to the knowledge of the regulations and encourage the presentation of their cases through PCIs. A form.[4] was generated on the website of the Coordination of Innovation and Biotechnology of the National Directorate of Bioeconomy called "Should my product be regulated?" which contains a short questionnaire that developers must complete to make inquiries on issues related to the regulation of GMOs or NBTs.[5]

Another action carried out by the Coordination of Innovation and Biotechnology of the National Directorate of Bioeconomy is the participation of local developers and representatives of the different public/private research institutions to attend virtual meetings (since they were held during the pandemic) with the aim of establishing a direct channel of communication with local developers in order to address relevant issues regarding problems related to regulation and funding that the institutions are currently facing.

Paying attention to the demands of local "Biodevelopers" to have a specific treatment from public policies, the "Argentine Biodevelopment Initiative" has been launched to accompany and strengthen capacities. This space seeks to promote innovation and accompany researchers and developers in the country in the management of activities related to biotechnology by promoting advances in regulatory processes. It seeks to facilitate and organize access to information and the generation of regulatory data to be presented to regulatory agencies.

International Cooperation

Argentina was the first country to develop specific regulations for the differential treatment of products derived from new breeding techniques. After the official publication of the first resolution in

[4]https://www.magyp.gob.ar/conabia/.
[5]https://www.magyp.gob.ar/conabia.

2015, other countries such as Chile.[6], Brazil.[7], Paraguay.[8] developed their regulations contemplating similar criteria. Subsequently, Colombia.[9], Guatemala and Honduras.[10] adopted similar regulatory frameworks, as well as Japan and Israel, which have their regulations in force.

Products derived from NBTs could be considered GMO or not, so there must be a prior analysis. The edge for being considered GM or no GM is the CPB definition of GMO or LMO. When a product derived from NBTs is not under the scope of the CPB GMO definition, in Argentina it is considered a conventional product. Taking the before mention into account, in those cases the Cartagena Protocol on Biosafety should not need to apply to genome editing as these are mutagenic techniques that don't require CPB oversight, as there is no "new genetic combination".

Currently, there are several international forums where Argentina actively participates along with other countries. One of the most important events was the participation in the formulation of two international declarations: in 2018, the International Declaration in favor of agricultural applications of precision biotechnology and in 2019 the South Agricultural Council (CAS) Declaration at the WTO (World Trade Organization) in favor of genomic editing techniques. Nevertheless, efforts are still needed in international dialogues, international capacity building and accurate promotion in order to properly adopt these technologies.

DISCUSSION

This regulation was a pioneer in analyzing products derived from biotechnology using NBTs. The approach based on the analysis of the product obtained (real cases) or to be obtained (hypothetical cases), instead of the technologies used (from the long and dynamic list of technologies called NBTs), allows the regulation to be kept up-to-date and can be applied regardless of the scientific advances that are presented.

The fact of having separated the analysis of NBTs from the rest of the GMO regulations is also a value generated in this update of the regulations. Developers find greater opportunities to approach the regulatory system and make their inquiries. This gives greater predictability to projects, especially those of local development, which always has many difficulties to complete the path of innovation with their products derived from the application of modern biotechnology.

The spirit of the regulation is to contemplate all organisms under the same resolution independently of commercial regulations for GMOs. Also, this regulation gives certainty to the local researchers and developers, and this is observed in the amount of developments and consultations carried out.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

MFG, AIW, PG, and DML contributed to write the manuscript.

REFERENCES

Barrangou, R., and Doudna, J. A. (2016). Applications of CRISPR Technologies in Research and Beyond. *Nat. Biotechnol.* 34, 933–941. doi:10.1038/nbt.3659

Burachik, M., and Traynor, P. L. (2002). *Analysis of a National Biosafety System: Regulatory Policies and Procedures in Argentina*. ISNAR Country Report 63. The Hague, the Netherlands: International Service for National Agricultural Research, 58. Available at: ftp://ftp.cgiar.org/isnar/Publicat/cr63.pdf

Chen, K., Wang, Y., Zhang, R., Zhang, H., and Gao, C. (2019). CRISPR/Cas Genome Editing and Precision Plant Breeding in Agriculture. *Annu. Rev. Plant Biol.* 70, 667–697. doi:10.1146/annurev-arplant-050718-100049

Duensing, N., Sprink, T., Parrott, W. A., Fedorova, M., Lema, M. A., Wolt, J. D., et al. (2018). Novel Features and Considerations for ERA and Regulation of Crops Produced by Genome Editing. *Front. Bioeng. Biotechnol.* 6, 79. doi:10.3389/fbioe.2018.00079

Eriksson, D., Kershen, D., Nepomuceno, A., Pogson, B. J., Prieto, H., Purnhagen, K., et al. (2019). A Comparison of the EU Regulatory Approach to Directed Mutagenesis with that of Other Jurisdictions, Consequences for International Trade and Potential Steps Forward. *New Phytol.* 222 (4), 1673–1684. doi:10.1111/nph.15627

Gavin, M. C., McCarter, J., Berkes, F., Mead, A. T. P., Sterling, E. J., Tang, R., et al. (2018). Effective Biodiversity Conservation Requires Dynamic, Pluralistic, Partnership-Based Approaches. *Sustainability* 10 (6), 1846. doi:10.3390/su10061846

González, M. N., Massa, G. A., Andersson, M., Turesson, H., Olsson, N., Fält, A.-S., et al. (2020). Reduced Enzymatic Browning in Potato Tubers by Specific Editing of a Polyphenol Oxidase Gene via Ribonucleoprotein Complexes Delivery of the CRISPR/Cas9 System. *Front. Plant Sci.* 10, 1649. doi:10.3389/fpls.2019.01649

ISAAA (2018). *Global Status of Commercialized Biotech/GM Crops in 2018: Biotech Crops Continue to Help Meet the Challenges of Increased Population and Climate Change*. Ithaca, NY: ISAAA. ISAAA Brief No. 54.

Knott, G. J., and Doudna, J. A. (2018). CRISPR-Cas Guides the Future of Genetic Engineering. *Science* 361 (6405), 866–869. doi:10.1126/science.aat5011

Lewi, D. M., and Vicién, C. (2020). Argentina's Local Crop Biotechnology Developments: Why Have They Not Reached the Market Yet? *Front. Bioeng. Biotechnol.* 8, 301. doi:10.3389/fbioe.2020.00301

Petracca, Y., Van Eenennaam, A. L., and Lema, M. (2016). Gene Editing: Do Not Forget about Animal Agriculture. *J. Adv. Res. Biotech.* 1 (1), 2. doi:10.15226/2475-4714/1/3/00109

[6]http://www.sag.cl/ambitos-de-accion/aplicabilidad-de-resolucion-ndeg-15232001-en-material-de-propagacion-desarrollado-por-nuevas-tecnicas-de-fitomejoramiento.

[7]http://ctnbio.mctic.gov.br/resolucoes-normativas/-/asset_publisher/OgW431Rs9dQ6/content/resolucao-normativa-n%C2%BA-16-de-15-de-janeiro-de-2018?redirect=http%3A%2F%2Fctnbio.mctic.gov.br%2Fresolucoes-normativas%3Fp_p_id%3D101_INSTANCE_OgW431Rs9dQ6%26p_p_lifecycle%3D0%26p_p_state%3Dnormal%26p_p_mode%3Dview%26p_p_col_id%3Dcolumn-2%26p_p_col_count%3D3%26_101_INSTANCE_OgW431Rs9dQ6_advancedSearch%3Dfalse%26_101_INSTANCE_OgW431Rs9dQ6_keywords%3D%26_101_INSTANCE_OgW431Rs9dQ6_delta%3D15%26p_r_p_564233524_resetCur%3Dfalse%26_101_INSTANCE_OgW431Rs9dQ6_cur%3D2%26_101_INSTANCE_OgW431Rs9dQ6_andOperator%3Dtrue.

[8]https://conbio.mag.gov.py/media/ckfinder/files/Resolucion%20565%20de%202019.pdf.

[9]https://www.ica.gov.co/getattachment/2d02cc52-d1c5-4123-8a5a-aea9ad2ce926/2018R29299.aspx.

[10]http://www.senasa.gob.hn/images/ACD/2019/ACUERDO-CD-SENASA-008-2019%20GACETA%2035047.PDF.

Whelan, A. I., Gutti, P., and Lema, M. A. (2020). Gene Editing Regulation and Innovation Economics. *Front. Bioeng. Biotechnol.* 8, 303. doi:10.3389/fbioe.2020.00303

Whelan, A. I., and Lema, M. A. (2015). Regulatory Framework for Gene Editing and Other New Breeding Techniques (NBTs) in Argentina. *GM Crops & Food* 6 (4), 253–265. doi:10.1080/21645698.2015.1114698

Whelan, A., and Lema, M. (2019). "Regulation of Genome Editing in Plant Biotechnology: Country Report – Argentina," in *Regulation of Genome Editing in Plant Biotechnology: A Comparative Analysis of the Regulatory Framework for Genome-Edited Plants in Selected Countries*. Editors H-G Dederer and D Hamburger (Heidelberg, New York, Dordrecht, London: Springer).

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The Handling Editor declared a past collaboration with one of the Authors DL at the time of review.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors, and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Bioengineering and Biotechnology

ORIGINAL RESEARCH
published: 25 February 2022
doi: 10.3389/fbioe.2022.843093

Should Gene Editing Be Used to Develop Crops for Continuous-Living-Cover Agriculture? A Multi-Sector Stakeholder Assessment Using a Cooperative Governance Approach

Nicholas R. Jordan[1], Jennifer Kuzma[2], Deepak K. Ray[3], Kirsten Foot[4], Madison Snider[4], Keith Miller[5], Ethan Wilensky-Lanford[5] and Gifty Amarteifio[4]*

[1]Agronomy and Plant Genetics, University of Minnesota, Saint Paul, MN, United States, [2]School of Public and International Affairs, Genetic Engineering and Society Center, NC State University, Raleigh, NC, United States, [3]Institute on the Environment, University of Minnesota, Saint Paul, MN, United States, [4]Department of Communication, University of Washington, Seattle, WA, United States, [5]Terraluna Collaborative, Minneapolis, MN, United States

OPEN ACCESS

Edited by:
Patricia Machado Bueno Fernandes, Federal University of Espirito Santo, Brazil

Reviewed by:
Muntazir Mushtaq, National Bureau of Plant Genetic Resources (ICAR), India
Tathiana Ferreira Sa Antunes, University of Florida, United States

***Correspondence:**
Nicholas R. Jordan
jorda020@umn.edu

Specialty section:
This article was submitted to Biosafety and Biosecurity, a section of the journal Frontiers in Bioengineering and Biotechnology

Received: *24 December 2021*
Accepted: *20 January 2022*
Published: *25 February 2022*

Citation:
Jordan NR, Kuzma J, Ray DK, Foot K, Snider M, Miller K, Wilensky-Lanford E and Amarteifio G (2022) Should Gene Editing Be Used to Develop Crops for Continuous-Living-Cover Agriculture? A Multi-Sector Stakeholder Assessment Using a Cooperative Governance Approach. Front. Bioeng. Biotechnol. 10:843093. doi: 10.3389/fbioe.2022.843093

Continuous-living-cover (CLC) agriculture integrates multiple crops to create diversified agroecosystems in which soils are covered by living plants across time and space continuously. CLC agriculture can greatly improve production of many different ecosystem services from agroecosystems, including climate adaptation and mitigation. To go to scale, CLC agriculture requires crops that not only provide continuous living cover but are viable in economic and social terms. At present, lack of such viable crops is strongly limiting the scaling of CLC agriculture. Gene editing (GE) might provide a powerful tool for developing the crops needed to expand CLC agriculture to scale. To assess this possibility, a broad multi-sector deliberative group considered the merits of GE—relative to alternative plant-breeding methods—as means for improving crops for CLC agriculture. The group included many of the sectors whose support is necessary to scaling agricultural innovations, including actors involved in markets, finance, policy, and R&D. In this article, we report findings from interviews and deliberative workshops. Many in the group were enthusiastic about prospects for applications of GE to develop crops for CLC agriculture, relative to alternative plant-breeding options. However, the group noted many issues, risks, and contingencies, all of which are likely to require responsive and adaptive management. Conversely, if these issues, risks, and contingencies cannot be managed, it appears unlikely that a strong multi-sector base of support can be sustained for such applications, limiting their scaling. Emerging methods for responsible innovation and scaling have potential to manage these issues, risks, and contingencies; we propose that outcomes from GE crops for CLC agriculture are likely to be much improved if these emerging methods are used to govern such projects. However, both GE of CLC crops and responsible innovation and scaling are unrefined innovations. Therefore, we suggest that the best pathway for exploring GE of CLC crops is to intentionally couple implementation and refinement of both kinds of innovations. More broadly, we argue that such pilot

projects are urgently needed to navigate intensifying grand challenges around food and agriculture, which are likely to create intense pressures to develop genetically-engineered agricultural products and equally intense social conflict.

Keywords: gene editing, agricultural diversification, multi-stakeholder, governance, cover crops

INTRODUCTION

Emerging biotechnologies such as gene editing may greatly advance critical frontiers in agricultural development, such as climate resilience or the welfare of resource-poor farmers and increase global food security (Karavolias Nicholas et al., 2021). However, society must also be protected from potential harmful effects—direct or indirect—of these biotechnologies on the environment, human health, or social welfare.

A pilot test of cooperative governance of gene editing (Jordan et al., 2017), applied to crops for continuous-living-cover agriculture, with a particular focus on cover crops, was conducted and is reported on here. Continuous-living-cover (CLC) agriculture integrates multiple crops to create diversified agroecosystems in which soils are continuously covered by living plant cover across time and space. Cover crops are an important element of CLC agriculture. By definition, cover crops are grown on farmland that would otherwise be fallow; these crops can enhance soil, water, and biodiversity in agricultural ecosystems by a wide range of mechanisms (Basche and DeLonge, 2017). So-called "cash cover crops" are a subset of cover crops which produce marketable agricultural commodities. In exploring the prospect of such applications of gene editing, this pilot project addressed matters of broad and global interest, as crops for CLC agriculture are widely seen as fundamental to progress on regeneration of degraded soils, which in turn is critical to sustaining agriculture productivity, water, biodiversity, and to climate adaptation and mitigation.

The initial stage of the cooperative governance pilot was a multi-sector, multi-stage deliberative process (Jordan et al., 2017). These deliberations included assessment of rewards and risks from potential applications of gene editing to cover crops, cash-cover crops, and other crops of particular value for CLC agriculture in temperate zones—such as the US Midwest region—where annual row crops now predominate. Deliberations also addressed governance, i.e., how such applications might be governed to manage inherent rewards and risks.

A key premise of the deliberative process is that any effort to use gene editing as a means for developing crops for CLC agriculture would succeed at scale only with multiple pillars of support, including development of markets for such crops, provision of finance, supportive policy, and social cohesion and collective action (per Herrero et al., 2020). Therefore, we recruited actors and stakeholders relevant to such sectors (e.g., markets, finance, policy, NGOs, think-tanks, farmers, trade organizations, industry, government, and academe) into the pilot project. We therefore consider our assessment of these applications of gene editing to be pragmatic, in the sense of being informed by the views and perceptions of actors and stakeholders that would be central to any effort to develop such crops *via* gene editing. Our project appears to be a relatively unique effort to convene and support a multi-stakeholder deliberative process around applications of GE to sustainable development of agriculture (see also Lotz et al., 2020). Implementation of such processes has been very limited, despite many calls for their use in governance of emerging biotechnologies (Kuzma, 2016; NAS, 2016; Jordan et al., 2017; Jasanoff and Hurlburt, 2018; Kofler et al., 2018; Montoliu et al., 2018; Resnik, 2018).

We note that at present, development and scaling of gene-edited crops of any sort is in early days. Certainly, assessment of the merits of gene editing should address presently evident risks and opportunities. However, any assessment of gene editing as a means of developing cover crops and cash cover crops must also be prospective and anticipatory, given the lack of actual experience. In particular, we suggest that it is necessary to enlarge the scope of assessment to encompass ***feasible methods for identification, assessment, and management of emerging rewards, risks, and societal impacts of gene editing applied to the crops of interest, as such applications go forward.*** We can anticipate, based on the history of scaling of innovations (Herrero et al., 2020; Wigboldus et al., 2020) that additional rewards, risks, and impacts will indeed emerge as the result of technological development, crop applications, scaling of resultant crops, and growing understanding of biophysical and social effects of these crops. Moreover, it is clear that broad stakeholder support for such applications is contingent on how emerging effects of applications are identified, assessed and managed (e.g., Gordon et al., 2021). Therefore, methods and capacities for managing the inherent dynamics and complexities of rewards, risks, and societal impacts are an important aspect of the use of gene editing to develop cover crops and cash cover crops.

Below, we outline key motivations for our pilot cooperative governance project, describe its initial deliberative phases, and report findings from interviews and workshops with participants and other actors, in the context of recent developments in governance of gene editing.

Motivations for Cooperative Governance of Gene Editing Applied to Crops for CLC Agriculture

Global Need for Diversified, Broadly-Regenerative Agriculture

Major transitions are needed in agriculture to create a broadly-regenerative agriculture, i.e., an agriculture that can remedy pervasive degradation of soil, water, and biodiversity, provide

climate-change adaptation and mitigation benefits, reduce diet-related health problems, and address inequity and injustice in agriculture and food systems (HLPE, 2019; Willett et al., 2019; Klerkx and Begemann, 2020; Rockström et al., 2020; Steiner et al., 2020). Diversification of current farm production systems appears fundamental to meeting these goals. Through a wide range of mechanisms, diversification can improve the condition of soil, water, and biodiversity resources (Lin, 2011; Kremen and Miles, 2012; Bowles et al., 2020; Tamburini et al., 2020), enable climate-change adaptation and mitigation, and support dietary shifts to lower the carbon intensity of human diets. Diversification also creates opportunities to enhance equity and other aspects of social sustainability, if socio-economic interventions that address these aspects are encompassed in diversification initiatives.

Diversification *via* Continuous Living Cover Agriculture

Several major diversification projects in agriculture rest on a concept of continuous living cover of farmland (Basche and DeLonge, 2017). These projects and initiatives are being implemented globally under a variety of banners, including "conservation agriculture," "soil health," and "regenerative agriculture," as the latter is most commonly framed (Lal, 2020), and have been strongly supported and advocated by public, private, and advocacy sectors. The common theme is regeneration of degraded soils as a means of enhancing agriculture productivity, water resources, biodiversity, and climate adaptation and mitigation. The essence of these projects is the design and scaling of agroecosystems that minimize soil disturbance and maximize the coverage of farmland with living crop-plant cover across the annual cycle (Jayaraman et al., 2021). In this concept of agroecosystem design and management, diversification is inherent because a range of crop and crop types (e.g., both annual, perennial) is necessary to achieve CLC across farmland and across the annual cycle. There is considerable evidence that agroecosystems based on CLC can support regeneration and provide climate adaptation and mitigation (Asbjornsen et al., 2008; Asbjornsen et al., 2014; Landis, 2017; Schulte et al., 2017; Brandes et al., 2018; King and Blesh, 2018; Burchfield et al., 2019).

CLC Agriculture Depends on Development of New Crops

Unfortunately, CLC agroecosystems often do not offer attractive short-term returns on investments (i.e., favorable cost/benefit ratios) (Plastina et al., 2020), or otherwise are economically feasible for only a subset of farmers (Giller et al., 2009). The unfavorable economics of CLC agroecosystems largely result from functional limitations of CLC crops—i.e., crops that can be used to increase continuous living cover in these agroecosystems. For example, in temperate-zone agriculture, fallow-season cover crops have received much attention in recent years. Such crops are planted in a fallow season after harvest of predominant crops (often summer annual crops such as maize and soybean) to conserve soil, water, and biodiversity, or to regenerate these elements of agroecosystems when their condition is degraded. By definition, these cover crops are not harvested for any marketable agricultural commodity. At the present time, use of such cover crops remains very limited in some major agricultural regions, such as the Midwest of North America (Rundquist and Cox, 2021), except when high levels of subsidies are provided (Rundquist and Cox, 2021).

Adoption appears to be reduced by functional limitations of these crops, which include limited germination, establishment, and early growth, nitrogen fixation, winter hardiness, slow biomass production and maturity, weed suppression, challenges in transition from cover crop to a subsequent crop, and limited seed production. Historically, cover crop breeding efforts have been very modest compared to dominant crops (Wayman et al., 2017); more comprehensive breeding programs are critically needed to reduce these functional limitations (Runck et al., 2020). In practice, these limitations are manifested as economic costs to farming operations that use cover crops.

One fundamental strategy for improving these economics is the development of "cash cover crops," as mentioned above. By definition, such crops provide both the agroecological benefits of cover crops, and yield valuable products for which scalable markets exist. A prime example of such a crop is camelina (*Camelina sativa*), which can serve as a cover crop while also showing high potential for many market opportunities (Zanetti et al., 2021). Most crops with high potential for such a dual-function role are novel or previously minor crops, and like camelina, many aspects of these crops need development to realize their potential. Specifically, development of these crops requires improved understanding of their genetics, genomics, and breeding; agronomic methods; agroecological interactions and effects; supply-chain infrastructure; and processing and product manufacturing. Therefore, a broad and robust program of crop breeding and development is needed to realize the potential of CLC agriculture. One such a program is the Forever Green Partnership, a broadly-based multi-sector/cross-scale collaboration that is working to develop CLC agriculture (Forever Green Partnership, 2021). Members of the Forever Green Partnership were the initial organizers of the pilot cooperative governance project, motivated by the project's interest in possible applications of gene editing in its development of CLC crops.

Crop-Breeding Strategies for Rapid Development of Crops for CLC Agriculture and Other Forms of Diversification

Integrative crop-breeding strategies are emerging for rapid genetic advancement of novel, previously minor, and "orphan" crops that can enable CLC agriculture and other forms of diversification. These strategies (e.g., Guilenque et al., 2020) integrate conventional breeding methods with genomic approaches that are based on DNA-sequence data obtained by the advent of rapid, inexpensive sequencing of whole genomes. Strategies can also include participatory breeding methods, in which farmers join as integral members of the breeding program

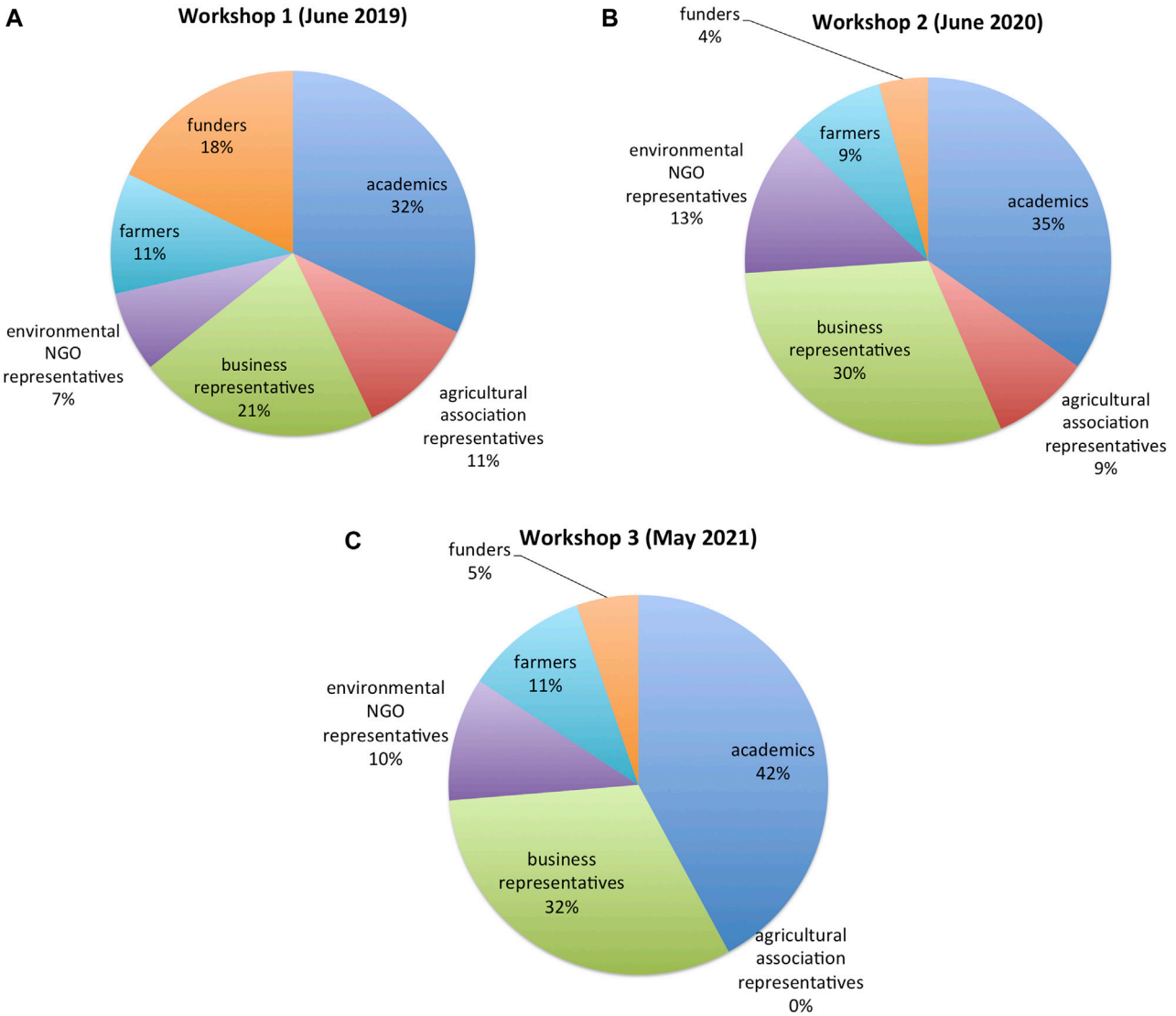


FIGURE 1 | Stakeholder participants in workshops in 2019 **(A)**; 2020 **(B)**; and 2021 **(C)**.

(Runck et al., 2014). For example, integrative breeding programs are being applied to a range of novel legume crops (Jiang, 2021).

The emerging technologies of gene editing may substantially accelerate development of new crops through integrative crop-breeding strategies. In particular, recent applications of gene editing to orphan crops in the genus *Physalis* suggest potential for rapid improvement in functional traits key to widespread commercialization. Crop breeders (Lemmon et al., 2018) envision that a range of orphan crops, currently important to smallholder agriculture in various regions globally (e.g., teff, grain amaranth, and cowpea) might be "catapulted into mainstream agriculture" by gene editing guided by genomic information from distantly related model crops. Advancing technical and methodological prospects for rapid genetic development of novel crops may provide a means for rapid development of crops for CLC agriculture.

However, public backlash towards 1st generation GM crops, generally used in predominant commodity crops such as maize, necessitates a careful examination of the societal concerns alongside the exploring of benefits of CLC and gene editing (Jordan et al., 2017; Kuzma, 2018). Therefore, our pilot cooperative governance project brought together a multi-sector group to explore the challenges and opportunities associated with CLC agriculture using gene editing.

METHODS

Cooperative Governance: Initial Deliberative Processes

As noted, we expect that GE applications to develop CLC crops will succeed at scale only with active support from a wide range of

societal sectors. Therefore, we recruited actors and stakeholders relevant to such sectors (including markets, finance, policy, NGOs, think-tanks, farmers, trade organizations, industry, government academe) into the pilot project and solicited input from additional subject-matter experts and stakeholders (SMESs) who were not formal participants in the pilot project. Recruitment was done by the lead organizer of the pilot project (Jordan), leveraging his professional networks, seeking participants for these sectors that were interested in joining the pilot project.

We organized two multi-day deliberative workshop gatherings for the multi-sector cooperative governance network, and a briefer capstone gathering at the end of the initial phase of the project which is ongoing. These workshops engaged a broad range of societal sectors (**Figure 1**), and were integral to the project's multi-sector cooperative governance approach to applications of GE to cover crops and "cash cover crops." The first gathering (2019) was in-person; 2020 and 2021 sessions were virtual, using an online meeting platform. The workshops were designed to facilitate deliberative engagement among project participants to assess the impacts (e.g., economic, environmental, and social) of such applications of gene editing. Importantly, the workshops also considered the possibility of taking collective action to address shared interests in the above governance, and particular options for operationalizing and implementing cooperative governance to manage inherent rewards and risks.

The first workshop was held in-person at the University of Minnesota over 3 days in June 2019. The 28 participants in the 2019 workshop included a wide range of sectors (**Figure 1A**) and the eight members of the pilot project organizing group, comprising six academics and two project evaluators. The second workshop convening was held online over 2 days in June 2020, with 23 participants from multiple sectors, and the same eight members of the project group (**Figure 1B**).

The initial workshop was designed to provide baseline knowledge about GE and cover crops, "cash cover crops," and other crops relevant to CLC agriculture, and to allow participants to exchange perspectives on these topics. The second workshop focused on deliberative discussion of scenarios of application and governance of GE. Scenarios were presented and discussed for two such crops: winter camelina and alfalfa. In small groups, participants discussed anticipated benefits and risks of such applications and several different scenarios for governance of these particular applications were discussed. These governance scenarios were developed by an online Delphi process that solicited participants' views on options for implementation of cooperative governance. These discussions set the stage for deliberations of a range of contrasting governance scenarios, and of prospects for implementation of one of these scenarios by the project group.

A third capstone workshop (May 2021) reviewed project activities, previewed remaining activities for the initial phase, and proposed a follow-up project for continued piloting of cooperative governance. The 2021 workshop was also held online, with 19 participants from multiple sectors, and the same eight persons from the project group (**Figure 1C**); its duration was 2 hours.

Semi-Structured Interviews

We used these interviews to elicit views of pilot-project participants at the project's inception and after the conclusion of the initial phase of the project. We also interviewed SMES who were not participating in the cooperative governance pilot project. The interviews were conducted prior to and after the three workshops, as shown in **Figure 2**.

Initial Interviews at Project Inception

Prior to the first cooperative governance workshop in June 2019, we conducted interviews with 30 participants in the cooperative governance pilot. Four of the initial interview participants were unable to attend the first workshop. The interviews focused on learning how cooperative governance participants viewed the major issues facing agriculture; understood the potential of CLC agriculture; perceived the risks and benefits of genomic editing; and had previously experienced cooperative governance.

The interview participants represented a diverse multi-sector network: 4 came from agricultural associations such as farmer organizations; 12 came from academic institutions engaged in crop, genome, or policy research; 1 came from an environmental NGO; 6 came from business and investment organizations; two were farmers; and 3 came from funding agencies. Two participants had unaffiliated designations.

Interviews were audio-recorded and transcribed for analysis. We used topic areas from the interview protocol instrument to develop a preliminary coding structure in the software MAXQDA, and coded by assigning a specific code to participant responses based on the question and the salient theme of their response. We then reviewed coded segments for each theme to provide comprehensive meaning and determine findings and insights of learning that support the stated purpose of the interview.

Interviews With Crop Breeders, Crop Geneticists, and Agroecologists

To complement viewpoints elicited in initial interviews with project participants, semi-structured, open-ended interviews were conducted with additional subject-matter experts, i.e., 17 geneticists and crop breeders, whose affiliations spanned academia, research institutes, and private companies; all were involved with development with crops for CLC agriculture. Semi-structured, open-ended interviews were also conducted with six agroecologists, whose affiliations included academia and NGOs. A few of these interviewees were full participants in the pilot project, but most were not. The goal of these interviews was to gain additional understanding of the perspectives of these sectors. For geneticists and breeders, interview questions addressed their views on the merits and drawbacks of GE as a means of developing cover crops, cash cover crops, and other crops for CLC agriculture, relative to alternative means such as "conventional" plant breeding, and the importance and urgency of developing such crops. For agroecologists, questions also queried views on the merits and drawbacks of GE as a means of developing crops for

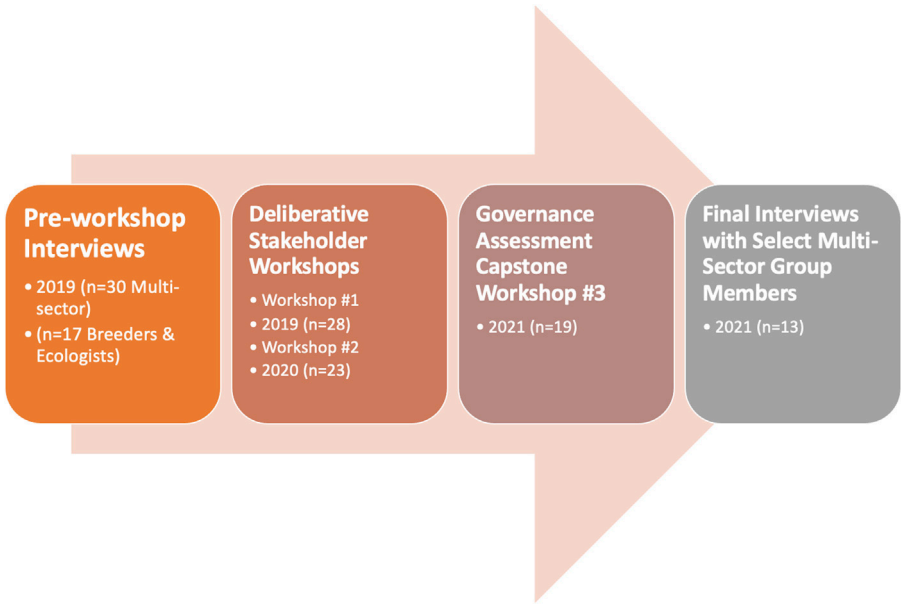


FIGURE 2 | Cooperative Governance Process: Initial Deliberative Stages. Note: for workshop participant affiliations see **Figure 1**. For interviewee affiliations, see Methods.

CLC agriculture, relative to alternative means such as "conventional" plant breeding. In addition, we elicited comment on anticipated environmental effects—desirable and undesirable—of widespread adoption of cover crops, cash cover crops, and other crops for CLC agriculture. Finally, we queried views on effects of such adoption of social sustainability. Interviewees were identified through professional networks and on the basis of interest in crops for CLC agriculture. All invitees agreed to an interview. Questions were provided in advance. All interviews were conducted by video or audio call and audio-recorded. Recordings were analyzed to summarize responses to questions, and frequently-expressed themes were identified and compiled.

Post-Workshop Interviews

These interviews explored participants' perspectives on GE as applied to crops for CLC agriculture, and how those perspectives have shifted over the past 2 years, during the duration of the pilot project. All 26 individuals that participated in the final cooperative governance workshop in 2021 (**Figure 1C**) were invited to a post-workshop interview. The 13 final interviews came from five different sectors: one farmer; two from agricultural organizations; three from environmental NGOs; three from academic institutions; and four from agricultural businesses.

These interviews encompassed several topics. First, we invited participants to talk about their perspectives on GE in regenerative agriculture. Second, we invited the participants to evaluate their experience in the pilot test of cooperative governance of GE. We also invited participants to express and discuss interest in participating in several options for a future second phase of the pilot test. Once the interviews were completed, the recorded files were transcribed for analysis as described in the pre-workshop interview section.

RESULTS AND DISCUSSION

Merits and Demerits of GE for Developing Crops for CLC Agriculture

We summarize (**Table 1**) themes from discussions of the multi-sector group and interviews subject-matter experts and stakeholders (SMESs) who were not participating in the cooperative governance pilot project. These themes encompassed views, reported below, on technical merits, agroecological effects, and societal impacts associated with CLC crop development by GE as compared to alternatives.

Technical Aspects of Plant Breeding and Germplasm Development

In our interviews with geneticists and breeders working on developing crops for regenerative agriculture, they were generally keen to use GE technology, because they saw much at stake. Specifically, our interviewees agreed that rapid development of new CLC crops was highly important, because of the large potential benefits of expanding CLC agriculture. Broadly, our interviewees were concerned about "'*missing out on environmental benefits*," such as reducing soil erosion, nutrient pollution of water, and coastal hypoxia. One participant suggested that without GE we would not be able to "*change the trajectory*" of "*grand challenges in agriculture/food/environment*." The key issue is that these geneticists/breeders consider new CLC crops—or existing crops that have received

TABLE 1 | Summary of themes from interviews: opportunities and challenges with gene editing and CLC agriculture.

Category	Opportunities (merits)	Challenges (demerits)
Technical Merits/ Demerits	• Greater potential to address grand challenges with agriculture using gene editing for CLC • Realize environmental benefits from CLC agriculture • Increased speed and efficiency of CLC crop improvement with gene editing	• Need to integrate gene editing with other breeding approaches • Competition with major commodity crops for funding and usage • Limited understanding of CLC crop genetics and tissue culture for gene editing to work • Lack of funding for CLC crop genetics and gene editing
Agroecological Aspects	• Potential to Improve soil quality, biodiversity, and management of crop pests, among other ecosystem services • With more rapid development of CLC crops through gene editing, more could be evaluated for ecosystem risks and benefits to select best for environment and diversified farming systems	• Potential ecosystem risks from CLC crops and gene editing • Possibility that CLC crops become monocultures if incentives for certain cash cover crops • Gene flow from CLC gene edited crops to wild relatives • Greater environmental movement of companion herbicides or pesticides used with gene edited cover crops
Societal Impacts	• Improvement of ecosystems and agricultural resilience • Possibility of developing more inclusive governance models around CLC agriculture and gene editing given early stages of field • Opportunity to increase public support through public and consumer value of CLC and gene editing	• R&D investments can be risky due to uncertain scalability • Costs of licensing of technology and regulatory compliance • Fear of over-commodification of CLC crops with gene editing due to investment need • Potential for greater inequality among farmers and harm to organic farmers • Fear of public opposition to gene editing in CLC agriculture

relatively little breeding effort, such as winter camelina—to need substantial genetic improvement, and therefore interrelated considerations of cost, speed, and efficiency of alternative breeding methods are of paramount importance. One of our interviewees noted that "*we could make a theoretical calculation regarding what is the impact of every year of delay on the problems that cover crops can address*." Without GE we would just "*bumble along with current methods* [and] *maybe won't achieve key goals*" and "*slow progress or block a viable path to production of a new crop*" with "*desirable traits*" in agronomic or product quality terms. Another described "*getting quite worried about getting the introgression traits and high yield if we don't use GE*" in cash cover crops.

In particular, geneticists/breeders voiced the need to combine improvement in both polygenic traits (e.g., yield) and key qualitative traits with simpler genetic control (e.g., functional traits of lipids) that are critical to commercialization of new crops. They expressed strong enthusiasm for combining GE of such qualitative traits with other breeding methodologies for polygenic traits. The key point is that simultaneous improvement was needed in both specific traits key to the commercialization of these crops and in a broader range of traits related to general adaptation of these advancing crops. Therefore, the ability to integrate breeding methods was seen as crucial. In general, the breeders and geneticists described the merits of GE in similar terms to those expressed in current accounts of GE (e.g., Jiang, 2021), such as precision of GE relative to alternative methods such as mutagenesis, and unique attributes of editing. They envisioned that, without GE, multiple decades of crop development would be needed to advance their CLC crops to the point of agronomic and commercial viability, whereas they might be able to advance crops to comparable viability within 5–10 years. Crucially, they were skeptical that crop development efforts could be sustained over multi-decade time frames that would be needed without GE, and therefore see GE as essential to development of a broad range of CLC crops. One expert noted that "*regenerative and cover crops have a lot to gain by using GE*" and "*given covid, economy, general turmoil—it will be easy for sustainability efforts in agriculture to fall by the wayside*" if "*we don't use GE* [thereby] *losing momentum*". Another noted that new crops face "*competition* [with] *major crops—corn, soybean—* [that] *are rapidly advancing in yield and productivity*" and "*we could miss our window*" and new "*crops will not be adopted which will be a shame*." The delay "*may turn into a barrier for companies not to adopt the crop. . .*".

However, geneticists/breeders emphasized that both knowledge of the fundamental biology of new crops and technical and methodological development are needed to apply GE to the crops they are working on. Specifically, interviewees pointed out that GE requires understanding of 'functional genomics' relevant to traits of interest, noting that GE technology "*works well if you know what changes need to be made*." Importantly, these breeders/geneticists emphasized that applications of GE to CLC crops requires understanding of genetic control of relevant plant phenotypes such as key product quality traits that are important to commercialization of new crops. Interviewees underscored the need for whole-genome sequencing of a reference genome and functional-biology understanding of GE targets as crucial prerequisites for GE applications to CLC crops. Interviewees also pointed out that technical and methodological development are needed to apply GE to some crops they are working on. For example, tissue-culture techniques needed to be developed for certain crops, and these were described as "*works-in-progress*." These breeders/geneticists also emphasized that considerable time and expense were required for development of both requisite

biological and genomic understanding and technical and methodological development, and that resources for development of CLC crops—regardless of particular methods—are quite limited at present. Therefore, investing these limited resources in developing technical and biological knowledge required for GE was perceived as risky by some.

Agroecological Effects

Participants in our multi-stage deliberative process, and agroecologists working on or strongly concerned with CLC agriculture and associated crops emphasized that development and broad adoption of CLC crops would provide many environmental benefits. The scope of these encompassed the full range of benefits of CLC crops that has been recognized by farmers and researchers, including improvements to soil fertility and quality, reductions in soil erosion and nutrient losses to surface and groundwater, enhancement of biodiversity in agroecosystems and agricultural landscapes, enhancement of total production and production of high-value commodities (e.g., novel sorts of proteins and lipids), farm profitability, and enhanced management of crop pests. Our interviewees underscored the low rates of adoption of conventional cover crops in the Midwest region and the wide range of agroecological problems associated with this lack of continuous living cover across the region. However, they also cautioned that problematic agroecological effects might result from widespread adoption of CLC crops.

Many of these concerns related to contingent effects that might result from any new-crop introduction into established agroecosystems. These include undesirable impacts on disease and arthropod pest dynamics, as might be affected by the practice of "planting green" (planting a crop into undecomposed cover crop residue), or by the persistent presence of cover crop residues soil processes, and nutrient cycling. There was concern that these effects were more likely if there were incentives for production of extensive monocultural stands of CLC crops.

Moreover, they noted potential tradeoffs associated with "cash cover crops," related to potential conflicts between commodity production and soil, water, and biodiversity conservation effects of cover cropping. Analogous concerns apply to CLC crops in general. Specifically, impacts of nutrient applications to enhance yield of CLC crops raised concerns of enhancing nutrient losses from agroecosystems. Also cover crops can deplete soil moisture in dry years, affecting subsequent crops. Fallow periods of uncovered soil may occur after harvest of cash cover crops.

Also, several concerns that are more particular to GE crops were noted as well. First, the "escape" from farms-- through wind, insect pollen vectors, and seed contamination—of GE plants, GE genes, and GE genomes was noted as a concern. Relevant impacts of such escape include introduction of genetic material into related wild or feral populations, potentially enhancing invasiveness of these populations, and into unedited or non-GMO crops. One expert noted that, previously, "*organic farmers were affected by pollen and pesticide drif*" from GMO crops and "*organic farmers who could no longer sell their produce as organic due to the cross over and were penalized. . .for something totally out of their control*".

Another concern relates to externalities such as off-farm movement of pesticides that might be triggered by adoption and scaling of GE crops. For example, extensive adoption GMO crops resistant to the herbicide dicamba has led to major off-farm impacts (Mortensen and Smith, 2020). In non-agricultural ecosystems, plant communities in these ecosystems have been altered by the herbicide, with significant harmful effects on biodiversity conservation in agricultural regions. Herbicide movement has also affected crops that are not resistant to the herbicide.

Essentially, our interviewees emphasized that any novel CLC crop—whether GE or not—might face significant agroecological barriers to scaling as noted above. There was concern that these effects were more likely if there were incentives for production of extensive monocultural stands, as might result if the market value of a CLC crop could be markedly enhanced by use of GE. Importantly, unanticipated and unintended agroecological "downsides" may manifest during the scaling of any new crop. Only ongoing monitoring can detect and manage such effects. Because of these potential limits on scalability, effective diversification of a regional cropping system is likely to require that a number of CLC crops will need to be introduced and evaluated; only some will be scalable in agroecological terms. Therefore, reducing the time and financial costs of CLC crop development is important, which on its face is an argument for using GE to rapidly advance a broad portfolio of CLC crops. On the other hand, it is important to recognize that any particular novel CLC crop may fail to establish at scale for agroecological reasons. This risk must be clear to all parties that invest in these crops, and particularly for the first CLC crops to which GE may be applied with the intent of commercial release of resultant crops.

Societal Aspects

Participants in the multi-stage process and our other informants were concerned with societal effects of GE of CLC crops. Generally, they saw potential for societal benefit through enhancement of the environmental performance of agroecosystems, and through enhancements in the range and resilience of agricultural production. However, they also were concerned about both procedural and distributive aspects of justice that might be associated with the development and scaling of gene-edited CLC crops.

Regarding the distribution of costs, benefits, and risks of development of GE CLC crop, many informants pointed to the costs of developing CLC crops with GE, considering licensing fees for the editing technologies, costs of regulatory compliance, and the need for capital investments in R&D that are inherently risky because of the uncertain scalability of new CLC crops. All of these factors were viewed as channeling developing of GE crops to well-capitalized private enterprise. Given this pathway for development of CLC crops by GE, our informants were mindful of potential tradeoffs of public goods for private interests. For example, skepticism was raised about applications of GE to produce conventional cover crops, which by definition do not produce marketable commodities. While these crops produce private benefits for farmers, e.g., *via* increasing soil

health and other agronomic benefits, they also arguably produce public goods related to soil, water, and biodiversity resources, and climate resilience. Our informants were doubtful that the economic value created by conventional cover crops would motivate private firms to invest in their development by GE. Commodity-producing CLC crops would therefore be the focus of private-sector GE development, raising concerns about tradeoffs between commodity production and other aspects of these crops, as noted above in the discussion of agroecological effects of CLC crops.

Further, it was noted the development of CLC crops will involve wealth creation—because "*cover crops need a whole supply chain and input industry . . . that is an economic development opportunity*." The question is: which scales of farmers and what kinds of farmers will benefit from development of CLC crops by GE? Will CLC crop development "*mainly support expansion in (large) systems, versus supporting middle and smaller operations?*" Concerns were also expressed about potential production of "product-linked" traits, such as the glyphosate tolerance of "Roundup-Ready" crops, in which the sales of the herbicide glyphosate are inherently promoted by development of such crops. Again, these were seen as opportunities for unwarranted value-capture by the private sector, with potential tradeoffs with public goods. For example, Roundup-Ready crops appear to have degraded a public good—the susceptibility of weeds to glyphosate. Our informants are concerned that if development of GE CLC crops is undertaken mainly by the private sector, such unintended and undesirable consequences may follow.

Informants also raised questions of procedural justice. For example, one informant (an agroecologist) articulated questions of "*who has power in our food system—and how will technologies change power dynamics and take power away from farmers.*" Another expert commented that "*high-value technology out on a field automatically sets up a situation where there is potential social injustice—from the standpoint of someone owning that technology—(with others) being denied the technology—or unable to afford it—or opposed to technology. . .(which) creates inequality among farmers.*" Another stated "*generally farmers appear to have little power regarding setting prices or structure of agriculture or ability to make changes. It seems that farmers have less and less power; Supply chains and end-use customers—they seem to have outsize power. . .historically, there has been little consideration of "fairness" to farmers.*" One informant voiced this concern about justice and trust of public research institutions: "*if the only way these developments can be implemented is by engagement of private sector, and there is a handoff to private sector . . . then this is very damaging to social contracts, including trust in public institutions and science.*" Moreover, "*we do not have precedent for open-source GMO technologies—something along those lines would alleviate potential injustices related to who does and who doesn't have access to technologies*".

Project participants and other informants also were concerned about the risk of losing the opportunity to develop and use GE technology for crop development if broad public opposition is aroused. This perceived risk was associated with several different scenarios of concern. First, some participants anticipated that critics of earlier GM crops were prepared to mount strong public campaigns against GE unless they were persuaded that objectionable aspects of those crops and their development will not be repeated in development of gene-edited crops. Secondly, some participants contended that a clear and compelling value proposition to the general public would be critical to avoiding broad public opposition to gene-edited crops. One participant observed "*If we continue to put out products that only bring value back to the farm. . ., I don't think it's necessarily going to change the paradigm that's out there, and I guess, what you're calling as the fear (.) It is a complete fear of the [GE] technology.*" Third, there was concern that mishaps in initial scaling of gene-edited crop—e.g., damage to organic crops by escape of genes from gene-edited crops—would damage the reputation of gene-edited crops in general. Risks associated with such scenario would affect crop developers, investors, and other parties with financial interests in development of particular gene-edited crops, but also may pose the general societal risk of reduced crop development during a time in which agriculture and food systems may face sharply mounting demands related to grand challenges.

The Use of GE to Develop Crops for Continuous-Living-Cover Agriculture: Social Sustainability and Risk Management Aspects

Below, we turn to a crucial consideration—governance and risk-management aspects of the use of GE to develop crops for CLC agriculture. Based on literature and our interviews with subject-matter experts and stakeholders (SMESs), we identify and discuss existing societal factors that are likely to pose challenges to the adoption, use, and success of gene-edited crops for CLC agriculture. We underscore that these societal factors are existing conditions and circumstances, constituting the current situation and context within which any near-term applications of GE will proceed. In essence, we highlight key aspects of the social "environment"—economic, cultural, political—that will affect adoption, use, and success of applications of GE to CLC crops. In their totality, we judge that this social environment is fraught with barriers and risks affecting successful outcomes from such applications. Therefore, it appears that barriers and risks must be adroitly and adaptively managed if applications of GE are to be successful in advancing the goals of CLC agriculture. It follows that a prospective assessment of the merits of GE for advancing these crops must consider prospects for managing these aspects.

First, we discuss in more detail some of the challenges associated with governance of CLC agriculture and GE (**Table 2**). Then, we conclude with a discussion about responsible innovation and scaling—based on governance and risk-management mechanisms that are more publicly robust and collaborative (Jordan et al., 2017; Kuzma, 2019; Kuzma and Grieger, 2020)—and how they are likely to be important to managing these barriers and risks, and thus enhancing

TABLE 2 | Summary of governance challenges associated with gene editing and CLC agriculture.

Category	Challenges	Possible remedies
Regulation	• Inability to trace some gene edited crops in CLC agriculture • Lack of harmonization for trade with EU • Rejection of gene editing by organic agriculture • Over or under-regulating with relation to cost or public confidence, respectively	• Responsible Innovation paradigm and Cooperative Governance models • Ensure robust regulation that is not too costly to small developers
Political Economy	• Limited investment in fallow season cover crops generally	• Combine sustainability benefits with production of valuable agricultural commodities to motivate investment in seed cost for farmers and R&D for seed producers
	• Financial risk with investment in CLC gene editing given uncertain scalability	• Develop scalability models
	• Navigating licensing, patents and ownership	• Assistance for small seed developers to navigate intellectual property
Public Acceptance/ Social License	• Fear of public opposition to gene editing in CLC agriculture • Lack of acceptance of gene editing community that public should have voice in governance	• Responsible Innovation paradigm and Cooperative Governance models • Better communication about the benefits of gene editing in CLC agriculture • Explore voluntary tracking and labeling schemes to ensure consumer choice

prospects for successful outcomes in applications of GE to CLC crops.

Factors Posing Challenges to Gene-Edited Crops for CLC Agriculture

Regulatory Landscapes

Systems of regulation and risk management relevant to gene-edited crops for CLC agriculture vary widely across nations, and create a wide range of challenges to exploration of these crops. In essence, current systems create barriers for poorly-capitalized developers of such crop, while also appearing to some observers as overly lax, arousing concerns for risk management. Finally, the variability of these systems across nations may enhance perceived risks for developers and investors.

Several first-generation GMO crops were regulated by relatively time-consuming regulatory processes. The regulatory impediment to gene-edited CLC crops is much lower in some global regions, but is much more stringent in others. However, even less-stringent regulatory processes may still pose a barrier to crop development efforts that have limited operating capital. Moreover, some civil-society groups consider that less-stringent processes are insufficient to manage public risks associated with GE crops, and these groups may take increasingly oppositional stances in the near future and some are already doing so on the basis of inadequate regulations under the recent 2020 USDA SECURE rule (see Center for Food Safety et al. v National Family Farm Coalition et al. v Vilsack).

Several GM CLC crops have been determined not to come under USDA's plant-pest regulations (USDA, 2020) and have been cleared for planting in agroecosystems. Some of these have been gene-edited. For example, seven lines of gene-edited pennycress were reviewed by USDA from 2018–2020 under the old "Am I Regulated"? (AIR) process (before the May 2020 USDA SECURE rule was passed). They were determined to fall outside of USDA's plant-pest regulations, and although USDA noted some concerns about pennycress being an agricultural weed (USDA, 2018), they were cleared for planting. The USDA's new SECURE rule grants automatic approval to gene-edited and other GM crops that have already been approved through the AIR process, and many newer gene-edited crops will also be exempt from regulation by USDA under SECURE (USDA, 2020). SECURE will trigger regulatory-review only if gene-edited developers introduce sequences that are not found in that species' gene pool. Exemptions may also be extended to genes coming from a sexually-compatible species.

Under SECURE, GM and gene-edited crops that are not automatically exempt will enter a screening stage called the Regulatory Status Review (RSR). USDA estimates 99% of GM crops will stop being reviewed by USDA after the RSR (Stokstad, 2020), and these crops would not require a publicly disclosed risk assessment, field trial, or permit (Kuzma and Grieger, 2020; USDA, 2020). Only the estimated 1% that pose a potential plant-pest risk would require a full risk assessment, permit for field trial, or any geographic restrictions. In summary, US regulation may not be the biggest barrier to gene-edited cover-crop development, given the SECURE exemptions and screening process for the RSR. One SMES noted that the GE "*regulatory process [is] much shorter*" in comparison to past GM crop regulation.

However, for some smaller companies and academic producers, the exemptions under SECURE and complex review pathways under SECURE may be difficult to navigate initially (Kuzma and Grieger, 2020). In the interviews, some SMESs expressed concern about regulation as a barrier to gene-edited cover-crop development. One noted "*the regulatory burden of GMOs was so high so that only seed companies with lots of capital can do transgenic events*." This perception may be due to the high cost estimates (circa $6 to $15M) for regulation of 1st generation GM crops (Kalaitzandonakes et al., 2007) done before the advent of the AIR process and SECURE rule. However, even these cost estimates often included molecular and agronomic characterization and other categories not directly related to safety assessments of GM crops (Kalaitzandonakes et al., 2007; Phillips, 2014). Furthermore, other estimates of regulatory costs for GM crops from public breeders and academics have been significantly less (Schiek et al., 2016). Even though regulatory costs may not be as high as gene-edited cover crop developers anticipate (Lassuoed et al., 2019),

small regulatory costs may nonetheless be prohibitive with limited investment in cover crop GE.

Other regulatory-related barriers to gene edited cover crops may be more important than the costs of going through the formal US regulatory system. First, the National Organic Standards Board has decided to exclude gene-edited crops from being certified as organic. This could create issues with the coexistence of organic versus non-organic farmers (such as those planting GE CLCs), cross-contamination through inadvertent comingling or gene flow leading to potential loss of markets for organic farmers, and segregation for different markets. A second related concern is that the EU and other countries have decided to regulate gene-edited crops more stringently and require labeling of gene-edited agricultural food products. With no formal regulation for most gene edited crops in the US and no labeling required for the vast majority of gene-edited foods under the new National Bioengineered Food Disclosure Standards (Jaffe and Kuzma, 2021), it will be nearly impossible to track gene-edited crops through the US food or feed supply (Kuzma and Grieger, 2020). The lack of traceability could create barriers to trade for farmers choosing to grow gene-edited cover crops and thus pose a financial risk from lost markets over concerns about cross contamination or comingled product streams. These concerns on domestic and global market may also make investors view investments in gene-edited crops, including cover crops, as more risky than conventional crops.

Political Economy

Developers of gene-edited crops for CLC agriculture require financial capital. However, many factors may limit availability of such capital, creating additional barriers for exploration of these crops. Concern about limited investment in fallow-season cover crops and the political economy of these crops was raised multiple times by the SMESs interviewed. The 1st generation of GM crops was dominated by large commodity crops like corn, soybeans, and cotton that now permeate US agricultural systems at over 93% total acreage (ISAAA, 2018). It was also dominated by large companies selling a high volume of GM crop seeds. In contrast, although cover crops grew in acreage by 50% from 2012–2017, they are incorporated on only 1.7% of US farmland (Runck et al., 2020; Rundquist and Cox, 2021). Cover crops are also usually planted to restore soil health, for weed control, or for other sustainability purposes. Worries about commercial investment in gene-edited cover crops thus seem warranted, given the history of 1st generation GM crops marked by large companies and seed sales' volume. SMESs whom we interviewed acknowledged the challenges with gene-edited cover crops in that it "*must be financially viable to grow the crop*" and "*cover crops are usually low value and low cost seed, [So] who will make the investment to improve a cover crop [with GE] that will continue to compete with low cost versions of the same crop?*"

Runck et al. (2020) discuss the need for a robust cover-crop seed industry that can provide affordable seed for producers, and they estimate that widespread US cover-crop adoption would require growing cover-crop seed on several million acres of cropland. Thus, cover-crop seed production would necessarily displace a proportion of the production of traditional cash crops. As such, economic incentives for cover crops would be needed, and if the cover crops were gene-edited, these incentives may become even more important to recoup the investment in laboratory R&D to produce them. Cover crops may decrease soil and chemical inputs needed for cash-crop production in alternate seasons and ultimately provide a net economic benefit to farmers, but whether this is enough of a financial benefit is unclear and will be context dependent.

Given these challenges to fallow-season cover cropping, CLC crops that combine sustainability benefits with production of valuable agricultural commodities may be necessary to motivate investment in seed cost for farmers and R&D for seed producers. For example, SMESs mentioned how GE could be used for a low lignin trait in alfalfa for "*happier cows producing more milk while eating less*" and improved "*camelina oil yield or quality . . . to get farmers a decent economic value proposition.*" These uses would have sustainability benefits to soil as cover crops as well as financial benefits to farmers.

Another economic issue for gene-edited cover crops is centered around ownership and intellectual property (IP). A SMES interviewee noted that "*with regard to I.P. for small companies this [technology] is very expensive. CRISPR patent is held by two groups and that is very expensive each time [for] a license fee.*" Montenegro deWit (2020) also found in her analysis that "despite the opening up of CRISPR IP for non-commercial research, CRISPR's commercial development remains tightly bound up in patents and licensing agreements." Another study noted with regard to gene-edited crops that "larger industry players. . .already appear to be more in control of the technology's agricultural and food applications" (Egelie et al., 2016). For example, DuPont Pioneer's gene-edited waxy corn is expected to be released into US markets under standard utility patent restrictions for one-time use (Montenegro de Wit, 2020). Licensing fees to develop gene-edited cover crops for commercial use may be prohibitive for smaller companies or public developers. Patented seeds for gene-edited cover crops could be prohibitively expensive if farmers are not able to commercialize or utilize products from them, in addition to reaping the sustainability benefits.

SMES interviewees summarized political economy concerns as "*producing a line and then introducing the plant in the field needs investment, and for cover crops, if companies do not have much interest they will not work on it, not invest in it.*" Even if companies are interested in investing in GE cover crops "*ownership of these technologies is an issue. It's dependent on profits, answerable to the shareholders. [So] how to build these technologies for the common good*" -- remains an outstanding question.

Public Acceptance ("Social License")

A majority of SMESs (plant breeders and geneticists) interviewed (13 of 17) expressed concern about public perception of gene-edited crops, including cover crops. Several of their comments fall into the "deficit model" thinking of public acceptance and communication. The "deficit model" assumes that a lack of public understanding or knowledge of science has led to the present skepticism toward science—that is, the public is assumed

to be "deficient" (of knowledge) while the scientific establishment is "sufficient" (in deserving a lack of skepticism, or in being trusted) (Sturgis and Allum, 2004). One SMES stated: "there will always be people who are scared by the technology—so education is as important as the technology", implying that if the public were educated, they would be less scared and more supportive of gene-edited crops. Other SMESs echoed this view with comments noting the *"really lousy job in introducing and educating public with GMOs"; that "a lot of social license restrictions [are] due to people not understand[ing] how the GM science works"; or that the real problem with the public was "their lack of knowledge*". Some dismissed public concerns about gene-edited crop risks as *"conspiracy theory is everywhere and people can create a climate of fear"*.

However, social science studies of public attitudes towards GM crops have shown that knowledge has modest and variable effects on public acceptance, with both positive and negative effects observed across multiple studies (Rose et al., 2019). In fact, researchers found those with higher levels of perceived familiarity are more concerned with GM foods, contradicting a main premise of the "deficit model" (Rose et al., 2019). In addition, other factors seem to be more important for public perception of GM crops such as trust in scientists and governments to manage risk, legitimacy of decision processes, respect for diverse cultural values and world views, and the public's ability to control their own exposure to risk or make their own choices about technological products (e.g. Siegrist et al., 2012; Yue et al., 2015a; also reviewed in; Kuzma, 2017).

In a recent public perception study comparing gene-edited to GM and conventional foods, researchers found that respondents viewed CRISPR and GM food similarly and substantially less positively than conventional food (Shew et al., 2018). The authors state that their study does not bode well for consumer acceptance of gene-edited foods. It is possible however that certain benefits can outweigh negative perceptions of GM and potentially gene-edited foods among some consumers, and there is support in the literature for the positive impact of specific benefits such as health, safety, and nutrition (Siegrist, 2008; Yue et al., 2015b). Several interviewees did mention that "convincing the public that these crops are beneficial" may help with public acceptance, but caution should be warranted with the attitude that "convincing" the public is the right approach. In light of other technological risk perception factors, engaging consumers and equipping them with information and choice seem better approaches to engendering trust and reducing skepticism towards gene-edited crops and foods.

Importantly, the possibility of public rejection of gene-edited crops creates major risks for all parties with a direct economic stake in their development. Crop developers and investors risk loss of financial capital and opportunity costs associated with development of gene-edited crops; advocacy organizations might lose reputational or political capital by endorsing GE crops that are subsequently rejected by the public. This complex of risk complicates the political economy of these crops.

The concept of "social license"—a notion borrowed from the mining industry—was mentioned by several interviewees and viewed to be important for the success of gene-edited cover crops. However, the utility of social license in biotechnology policy has been criticized, because the concept entails a limited scope of public engagement (Delborne et al., 2020). Social license implies that scientists and decision makers need only to ask for public permission once (a license) after technologies have already been defined and assessed by expert communities (Delborne et al., 2020). In contrast, meaningful public engagement would include stakeholders and publics in the formulation of problems to address with GE, in defining endpoints for risk assessment, and in continual monitoring and re-evaluation of gene-edited products in the face of uncertainties and complexities of releasing them into agroecosystems. One SMES suggested a solution closer to meaningful public engagement by stating that *"a collective/broad group could develop a scale on which individual [GE] technologies could be weighed to see how they effect a community"*. Calls for community engagement in GE and decision making have been made by several researchers and scholars (e.g., Jordan et al., 2017; Kofler et al., 2018; Kuzma and Grieger, 2020).

Current Approaches to Governance

Unfortunately, gene-edited crop developers are repeating mistakes in governance that occurred with the 1st generation of GM crops and foods, which may increase risk and costs associated with the use of GE to advance crops for CLC agriculture.

First, developers continue to take somewhat contradictory stances and make unsubstantiated claims about the technology and regulation (Kuzma et al., 2016; Kuzma, 2018; Bain et al., 2020) that public critics have recognized and critiqued in the past. Developers tend to communicate that although GE is a phenomenal technological leap that shows great promise, it is nothing new in comparison to conventional breeding and should therefore not undergo regulation (Kuzma et al., 2016; Kuzma, 2018; Bain et al., 2020; Qaim, 2020). This hypocrisy has been detected and noted by various publics in past controversies. Also, overpromising that GE is necessary for a second green revolution (Bain et al., 2020) may engender public mistrust, as 1st generation GM crops did not appreciably increase yields on average (Gould et al., 2017).

Second, consumers generally want to know that technological products are being regulated and the scope of governance includes potential health and environmental risks. Yet, many gene-edited crop developers have taken the stance that these crops should not be regulated. The lack of oversight, or a failure to minimize harm (e.g., USDA will not screen for off-target edits or regulate based on weediness risks Kuzma and Grieger, 2020), may jeopardize public confidence.

Third, as regulatory processes are developing in the US, it appears that there will be a lack of transparency about what gene-edited crops are being reviewed and how they are regulated (Kuzma, 2018; Kuzma and Grieger, 2020). In addition, most gene-edited foods will not be labeled (Kuzma, 2018). Without labeling, consumers do not have access to information to make their own informed decisions, which takes control away from them in determining their own exposure to risks, however small they may be. Ample risk perception studies indicate that people

view risks they cannot control as higher than those they can (e.g., Slovic, 1987). There are also efforts to obfuscate terminology—new USDA labeling standards do not use the term GM (instead use bioengineered) and gene-edited crop developers use terms like "new plant breeding technology" (NPBT). With the 1st generation of GM foods, consumers were largely unaware that they were eating them for years, and now there is a concerted backlash against them in the marketplace as organic and non-GM markets grow. Consumers may feel tricked by differential terminology and the lack of transparency, should they be able to find out that GE is a derivative of modern biotechnology, and trust in GE industries would be difficult to restore (Kuzma, 2018).

Finally, our regulatory system for gene edited crops is based on a narrow set of direct health and environmental risks. Yet, consumers care also about indirect ecosystem risks and benefits (e.g., climate change or resource use); health risks such as food allergenicity or sensitivity from low level consumption over a lifetime; social, economic, and cultural impacts; procedural justice and social equity; respect for nature; and ethical dimensions of rights to know and choose. For decades with GM crops, these broader societal aspects were marginalized and there is no space for legitimate discussion of them as scientists were adamant about "science-based regulation" (which almost exclusively addressed direct health or environmental risks that could be shown in laboratory-based toxicity studies). There has been a lack of respect for concerns voiced that are outside of the narrow purviews of the regulatory agencies (Thompson et al., 2007; Kuzma, 2018). Power and voice are given to a narrow set of technical experts, largely those of the product developer and regulatory staff (as public federal advisory committee processes have been lacking recently for gene edited products) (Kuzma, 2018). Yet, procedural fairness is an important factor for public acceptance of GM crops (Siegrist et al., 2012).

In summary, systemic barriers to exploration of gene-edited crops for CLC agriculture are being created by the regulatory landscape, political economy, public acceptance, and current governance approaches to gene-edited crops. If these barriers are not proactively addressed, we suspect these barriers will greatly slow exploration of these crops. Below we discuss responsible innovation and governance models that may enhance public trust, procedural legitimacy, and public confidence in gene-edited cover crops, and which may therefore be necessary adjuncts to the GE technologies themselves.

Shared Governance and Robust Risk Management: Key Support Pillars for Development of New Crops for CLC Agriculture?

In this section, we draw on insights from the CG pilot project and literature, but also our experience with GM crop governance over the past 30 years, observations of the field, and normative conclusions from these observations and experiences. We propose that societal adoption and acceptance of gene-edited crops for CLC agriculture—if these are to occur—may require replacing outdated notions of "deficit model thinking" and "social license" with more collaborative and publicly-robust governance processes.

Towards this end, a range of models for governance of gene-edited crops have been proposed. One example, as noted previously, is the Jordan et al. (2017) Cooperative Governance model for gene-edited cover crops. This model, piloted in the current project, engages a network of multiple subject matter experts, stakeholders, and investors in decision making about whether to move forward with a gene-edited cover crop. The multi-stakeholder cooperative governance group would conduct a comprehensive, multi-criteria assessment of the relative risks and benefits of a gene-edited crop designed for a specific purpose or environment. The group would also consider societal, economic, and cultural aspects before deciding to move forward with the gene-edited cover crop. Investors would mitigate risk by investing in crops that underwent such a rigorous evaluation by the diverse group.

Kuzma and Grieger (2020) recently proposed a "community-led and responsible governance" (CLEAR-GOV) model for gene-edited crops that would center on a repository of information about what gene-edited crops enter agroecosystems and food markets and a certification process to incentivize the sharing of such information. They note the lack of public information for many exempt crops under the new SECURE rules and the lack of labeling and traceability in discussing the need for such a repository. A multi-stakeholder advisory group, in concert with a public advisory group and crop-developer input, would guide the information required to be certified, the structure of the repository and the balancing of public information with IP protection and privacy. At a minimum the host plant, growth environment, purpose of the trait, and potential uses should be deposited. They argue that such transparency is more likely to engender public and consumer trust.

Some developers of gene–edited crops are working with the non-profit Center for Food Integrity (CFI) and an associated multi-stakeholder coalition to draft guidelines for responsible use of GE (The Centre Food Integrity, 2020). This model entails voluntary stewardship certification, intiated by GE developers self-assessing themselves against a checklist of best practices; a verification group then reviews self-assessments. Developers would have discretion to conceal edited plant varieties and traits as confidential business information, and no central repository of gene-edited crops and traits would be maintained.

Finally, Kuzma (2019) described a more open and "procedurally robust" risk assessment framework for transgenic organisms. This framework highlights that risk analysis is laden with assumptions and interpretations based on values. For example, the endpoints chosen in a risk assessment are based on what involved stakeholders care about (e.g., certain species, certain products of agriculture, or certain natural resources, etc.). Science gives us a guide, but what risks are acceptable are based on values, taking into consideration particular experiences, culture, perceptions of benefits, control over the situation, and trust in those managing risks (Kuzma, 2017). When new biotechnology products are initially released

into ecosystems, evaluating the "substantive validity" of risk assessments –where outcomes of the risk assessment are compared to what happens in reality—is not generally feasible, especially prior to any environmental release. Therefore, "procedural validity" of the risk assessment—i.e., how the risk assessment is conducted—becomes even more important than attempting to ascertain the substantive validity of particular risk evaluations prior to initial release and monitoring.

Following this reasoning, Kuzma (2019) outlined a framework for conducting robust risk analysis in support of formal regulatory decision making: the "Procedurally Robust Risk Analysis Framework" (PPRAF). The framework draws upon "responsible innovation" principles of humility, procedural validity, inclusion, anticipation, and reflexivity. PPRAF call on risk analysis to acknowledge uncertainty, engage multiple interested and affected parties in a holistic discussion of ends and means of innovation and associated risks; anticipate future conditions and contigencies; and promote mutual learning and reflection on the transparency, openness, and procedural validity of the risk analysis, and of uncertainty associated with conclusion.

The above governance and risk assessment models cannot guarantee public acceptance, but they are more likely to engender legitimacy and trust. Trust in government or experts to manage technologies has been a factor identified as a key factor for public acceptance of technologies (e.g., Siegrist et al., 2012; Yue et al., 2015a; Yue et al., 2015b).

Prospects for Implementation of Robust and Responsible Governance and Risk Assessment Models

Our deliberative workshops identified a moderately broad shared interest, among a multi-sector group, in several potential models for robust and responsible governance and risk assessment. Two of these models can be outlined as follows.

Stakeholder Governance: Deliberative foresight assessment by a broad and diverse range of stakeholders to evaluate social, scientific, economic, and cultural impacts, both positive and negative, of gene-edited crops. Crop developer decides on crop release. The crop would be certified for "inclusive stewardship."

Community Governance: Deliberative foresight assessment to evaluate crops by a broad and diverse range of interested and affected people, e.g., community groups including marginalized and indigenous communities and organic farmers. Consensus or majority decision regarding crop release. The crop would be certified for "responsible development and community approval."

However, there was a considerable range of opinion about the merits of these models, and some robust disagreement during the deliberative process. We also note that biotechnology industry innovators (Roberts et al., 2020) were found to be skeptical of the practicality of such responsible innovation and governance methods, particularly on the basis of perceived time demands and concern that such methods will make the innovation process too slow. Consequently, we recommend an exploratory application of these method in a particular pilot situation, and are pursuing that in current stages of our pilot governance project.

CONCLUSION

We found that a group of plant breeders developing cover crops and other crops for enhancing productive living cover in agriculture are very eager to use GE as a tool for developing CLC crops. A group of agroecologists working on development of diversified agroecosystems are strongly committed to enhancing CLC agriculture, and see merit in applying GE as a tool for developing relevant crops. However, the agroecologists also have a number of concerns about potential environmental consequences of applications of GE. Generally, these consequences are typical of agronomic and agroecological effects that can accompany any diversification of an agroecosystem, e.g., new pest problems, potential escape of invasive feral populations of a new crop, or changes in nutrient cycling.

Like plant breeders and agroecologists, other participants in the cooperative governance pilot projects also expressed generally positive views of GE as a means of developing new crops for diversification of agriculture. However, project participants from many sectors have concerns about societal impacts of applications of GE. These concerns center on procedural and distributional justice issues—who will govern the applications of GE, and how will benefits resulting from successful development and scaling of these crops be distributed? Likewise, how will costs and risks associated with the scaling of these crops be distributed. How will these applications of GE be governed, and what groups or parties will have power and influence in governance? More broadly, what kind of agriculture will result from applications of GE?

Broadly, it appears that responsible innovation and scaling practices and approaches will be necessary to address these concerns. In essence, the current situation features many social factors that pose challenges to applications of GE to advance CLC crops. First, the regulatory landscape is complex, varying markedly across global regions, and creating dilemmas and moral hazards for crop developers that may strongly limit the development, adoption, and use of GE CLC crops. Secondly, there are significant political-economic barriers to development of CLC crops, which will take concerted cross-sector action to surmount. Thirdly, current governance and risk-management approaches risk triggering strong opposition by civil-society groups. Sustained use of responsible innovation and scaling practices and approaches may surmount these barriers. At present, the willingness of a broad range of societal actors to participate in sustained responsible innovation and scaling processes is very unclear. Relevant actors have little experience with responsible innovation and scaling approaches, and therefore additional pilot projects are urgently needed.

The need for additional piloting of responsible innovation and scaling is particularly urgent because the current status quo may drive a dynamic of increasing uncertainty and opposition to use of GE, amplified by the stances of powerful food system actors such as CPG firms, which currently appear largely unwilling to publicly discuss potential applications of GE. If continued, we expect that this dynamic will greatly inhibit investment and

exploration of GE for development of CLC agriculture or other forms of diversified agriculture. Specifically, to advance and scale, crops for CLC agriculture must attract the "innovation accelerators" highlighted by Herrero et al. (2020), including finance, supportive policy, markets, ongoing R&D, and concerted cross-sector collective action, to both advance and scale these crops and to govern their development. If the status quo continues, emerging CLC crops appear unlikely to attract the requisite innovation accelerators or pillars of support needed have impact at scale and to reward public and private investment.

In closing, we suggest that is it is now essential to approach GE not as a standalone innovation, but rather as an element of "socio-technological innovation bundles" (Barrett et al., 2020). Such "bundles" are systems that include GE coupled variously with other relevant innovations that are broadly social in nature, e.g., in responsible innovation and scaling, and perhaps in other aspects, such as novel cooperative business and crop stewardship structures (Gary, 2019) and finance innovations such as Environmental, Social, and Governance Investing (ESG, Tucker and Jones, 2020). In our view, GE coupled to responsible innovation and scaling and other innovations appears to have high potential to attract broad societal support and could be applied widely in development of new crops to address agricultural diversification and related grand challenges. In the absence of such coupling, such use of GE may entail larger risks for crop developers and encounter strong societal opposition.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors upon request, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Minnesota Institutional Review Board. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

All authors contributed to study design; all authors participated in analysis and interpretation of interview and/or survey data; all authors reviewed and commented on drafts of the articles, NJ, DR, and JK developed initial draft.

FUNDING

This project was supported by a grant from the Walton Family Foundation, 2019–1163. Funds for publication fees were provided by the University of Minnesota-Twin Cities.

REFERENCES

Asbjornsen, H., Hernandez-Santana, V., Liebman, M., Bayala, J., Chen, J., Helmers, M., et al. (2014). Targeting Perennial Vegetation in Agricultural Landscapes for Enhancing Ecosystem Services. *Renew. Agric. Food Syst.* 29, 101–125. doi:10.1017/s1742170512000385

Asbjornsen, H., Shepherd, G., Helmers, M., and Mora, G. (2008). Seasonal Patterns in Depth of Water Uptake under Contrasting Annual and Perennial Systems in the Corn Belt Region of the Midwestern U.S. *Plant Soil* 308, 69–92. doi:10.1007/s11104-008-9607-3

Bain, C., Lindberg, S., and Selfa, T. (2020). Emerging Sociotechnical Imaginaries for Gene Edited Crops for Foods in the United States: Implications for Governance. *Agric. Hum. Values* 37, 265–279. doi:10.1007/s10460-019-09980-9

Barrett, C. B., Benton, T. G., Fanzo, J., Herrero, M., Nelson, R. J., Bageant, E., et al. (2020). *Socio-technical Innovation Bundles for Agri-Food Systems Transformation, Report of the International Expert Panel on Innovations to Build Sustainable, Equitable, Inclusive Food Value Chains*. Ithaca, NY, and London: Cornell Atkinson Center for Sustainability and Springer Nature.

Basche, A., and DeLonge, M. (2017). The Impact of Continuous Living Cover on Soil Hydrologic Properties: a Meta-Analysis. *Soil Sci. Soc. America J.* 81, 1179–1190. doi:10.2136/sssaj2017.03.0077

Bowles, T. M., Mooshammer, M., Socolar, Y., Calderón, F., Cavigelli, M. A., Culman, S. W., et al. (2020). Long-term Evidence Shows that Crop-Rotation Diversification Increases Agricultural Resilience to Adverse Growing Conditions in North America. *One Earth* 2, 284–293. doi:10.1016/j.oneear.2020.02.007

Brandes, E., McNunn, G. S., Schulte, L. A., Muth, D. J., VanLoocke, A., and Heaton, E. A. (2018). Targeted Subfield Switchgrass Integration Could Improve the Farm Economy, Water Quality, and Bioenergy Feedstock Production. *GCB Bioenergy* 10, 199–212. doi:10.1111/gcbb.12481

Burchfield, E. K., Nelson, K. S., and Spangler, K. (2019). The Impact of Agricultural Landscape Diversification on U.S. Crop Production. *Agric. Ecosyst. Environ.* 285, 106615. doi:10.1016/j.agee.2019.106615

Delborne, J. A., Kokotovich, A. E., and Lunshof, J. E. (2020). Social License and Synthetic Biology: the Trouble with Mining Terms. *J. Responsible Innovation* 7, 280–297. doi:10.1080/23299460.2020.1738023

Egelie, K. J., Graff, G. D., Strand, S. P., and Johansen, B. (2016). The Emerging Patent Landscape of CRISPR-Cas Gene Editing Technology. *Nat. Biotechnol.* 34, 1025–1031. doi:10.1038/nbt.3692

Forever Green Partnership (2021). https://forevergreenpartnership.umn.edu/.

Gary, S. N. (2019). The Oregon Stewardship Trust: A New Type of Purpose Trust that Enables Steward-Ownership of a Business. *Univ. Cincinnati Law Rev.* 88, 707.

Giller, K. E., Witter, E., Corbeels, M., and Tittonell, P. (2009). Conservation Agriculture and Smallholder Farming in Africa: The Heretics' View. *Field Crops Res.* 114, 23–34. doi:10.1016/j.fcr.2009.06.017

Gordon, D. R., Jaffe, G., Doane, M., Glaser, A., Gremillion, T. M., and Ho, M. D. (2021). Responsible Governance of Gene Editing in Agriculture and the Environment. *Nat. Biotechnol.* 39, 1055–1057. doi:10.1038/s41587-021-01023-1

Gould, F., Amasino, R. M., Brossard, D., Buell, C. R., Dixon, R. A., Falck-Zepeda, J. B., et al. (2017). Elevating the Conversation about GE Crops. *Nat. Biotechnol.* 35, 302–304. doi:10.1038/nbt.3841

Guilengue, N., Alves, S., Talhinhas, P., and Neves-Martins, J. (2020). Genetic and Genomic Diversity in a Tarwi (Lupinus Mutabilis Sweet) Germplasm Collection and Adaptability to Mediterranean Climate Conditions. *J. Agron.* 10, 21.

Herrero, M., Thornton, P. K., Mason-D'Croz, D., Palmer, J., Benton, T. G., Bodirsky, B. L., et al. (2020). Innovation Can Accelerate the Transition towards a Sustainable Food System. *Nat. Food* 1, 266–272. doi:10.1038/s43016-020-0074-1

HLPE (2019). *Agroecological and Other Innovative Approaches for Sustainable Agriculture and Food Systems that Enhance Food Security and Nutrition: A Report by the High Level Panel of Experts on Food Security and Nutrition of the Com- Mittee on World Food Security*. Rome

Isaaa (2018). *Brief 54-2018: Executive Summary; Biotech Crops Continue to Help Meet the Challenges of Increased Population and Climate Change*. Available at: https://www.isaaa.org/resources/publications/briefs/54/executivesummary/default.asp

Jaffe, G., and Kuzma, J. (2021). New Bioengineered (Aka GM) Food Disclosure Law: Useful Information or Consumer Confusion? Food Drug and Law Institute Magazine. Summer 2021 Available at: https://www.fdli.org/2021/04/new-bioengineered-aka-gm-food-disclosure-law-useful-information-or-consumer-confusion/.

Jasanoff, S., and Hurlbut, J. B. (2018). A Global Observatory for Gene Editing. *Nature* 555, 435–437. doi:10.1038/d41586-018-03270-w

Jayaraman, S., Dang, Y. P., Naorem, A., Page, K. L., and Dalal, R. C. (2021). Conservation Agriculture as a System to Enhance Ecosystem Services. *Agriculture* 11, 718. doi:10.3390/agriculture11080718

Jiang, G.-L. (2021). Special Issue "Genetics, Genomics, and Breeding of Legume Crops". *Agronomy* 11, 475. doi:10.3390/agronomy11030475

Jordan, N. R., Dorn, K. M., Smith, T. M., Wolf, K. E., Ewing, P. M., Fernandez, A. L., et al. (2017). A Cooperative Governance Network for Crop Genome Editing. *EMBO Rep.* 18, 1683–1687. doi:10.15252/embr.201744394

Kalaitzandonakes, N., Alston, J. M., and Bradford, K. J. (2007). Compliance Costs for Regulatory Approval of New Biotech Crops. *Nat. Biotechnol.* 25, 509–511. doi:10.1038/nbt0507-509

Karavolias Nicholas, G., Wilson, H., Abugu Modesta, N., and Evanega Sarah, N. (2021). Application of Gene Editing for Climate Change in Agriculture. *Front. Sustain. Food Syst.* 5, 296. doi:10.3389/fsufs.2021.685801

King, A. E., and Blesh, J. (2018). Crop Rotations for Increased Soil Carbon: Perenniality as a Guiding Principle. *Ecol. Appl.* 28, 249–261. doi:10.1002/eap.1648

Klerkx, L., and Begemann, S. (2020). Supporting Food Systems Transformation: The what, Why, Who, where and How of mission-oriented Agricultural Innovation Systems. *Agric. Syst.* 184, 102901. doi:10.1016/j.agsy.2020.102901

Kofler, N., Collins, J. P., Kuzma, J., Marris, E., Esvelt, K., Nelson, M. P., et al. (2018). Editing Nature: Local Roots of Global Governance. *Science* 362, 527–529. doi:10.1126/science.aat4612

Kremen, C., and Miles, A. (2012). Ecosystem Services in Biologically Diversified versus Conventional Farming Systems: Benefits, Externalities, and Trade-Offs. *E&S* 17, 40. doi:10.5751/ES-05035-170440

Kuzma, J., and Grieger, K. (2020). Community-led Governance for Gene-Edited Crops. *Science* 370, 916–918. doi:10.1126/science.abd1512

Kuzma, J., Kokotovich, A., and Kuzhabekova, A. (2016). Attitudes towards Governance of Gene Editing. *Asian Biotechnol. Dev. Rev.* 18, 69–92.

Kuzma, J. (2016). Policy: Reboot the Debate on Genetic Engineering. *Nature* 531, 165–167. doi:10.1038/531165a

Kuzma, J. (2019). Procedurally Robust Risk Assessment Framework for Novel Genetically Engineered Organisms and Gene Drives. *Regul. Governance* 15, 1144–1165. doi:10.1111/rego.12245

Kuzma, J. (2018). Regulating Gene Edited Crops. *Issues Sci. Technol.* 35, 80–85.

Kuzma, J. (2017). "Society and Policy Makers' Responsibilities," in *Consumer Perception of Product Risks and Benefits*. Editors G. Emilien, R. Weitkunat, and F. Luedicke (Dordrecht: Springer).

Lal, R. (2020). Regenerative Agriculture for Food and Climate. *J. Soil Water Conservation* 75, 123A–124A. doi:10.2489/jswc.2020.0620a

Landis, D. A. (2017). Designing Agricultural Landscapes for Biodiversity-Based Ecosystem Services. *Basic Appl. Ecol.* 18, 1–12. doi:10.1016/j.baae.2016.07.005

Lassoued, R., Phillips, P. W. B., Smyth, S. J., and Hesseln, H. (2019). Estimating the Cost of Regulating Genome Edited Crops: Expert Judgment and Overconfidence. *GM Crops & Food* 10, 44–62. doi:10.1080/21645698.2019.1612689

Lemmon, Z. H., Reem, N. T., Dalrymple, J., Soyk, S., Swartwood, K. E., Rodriguez-Leal, D., et al. (2018). Rapid Improvement of Domestication Traits in an Orphan Crop by Genome Editing. *Nat. Plants* 4, 766–770. doi:10.1038/s41477-018-0259-x

Lin, B. B. (2011). Resilience in Agriculture through Crop Diversification: Adaptive Management for Environmental Change. *J. Biosci.* 61, 183–193. doi:10.1525/bio.2011.61.3.4

Lotz, L. A., van de Wiel, C. C., and Smulders, M. J. (2020). Genetic Engineering at the Heart of Agroecology. *Outlook Agric.* 49 (1), 21–28. doi:10.1177/0030727020907619

Montenegro de Wit, M. (2020). Democratizing CRISPR? Stories, Practices, and Politics of Science and Governance on the Agricultural Gene Editing Frontier. *Elementa-Sci Anthrop* 8, 9. doi:10.1525/elementa.405

Montoliu, L., Merchant, J., Hirsch, F., Abecassis, M., Jouannet, P., Baertschi, B., et al. (2018). ARRIGE Arrives: Toward the Responsible Use of Genome Editing. *CRISPR J.* 1, 128–129. doi:10.1089/crispr.2018.29012.mon

Mortensen, D. A., and Smith, R. G. (2020). Confronting Barriers to Cropping System Diversification. *Front. Sustain. Food Syst.* 4, 199. doi:10.3389/fsufs.2020.564197

NAS (2016). *Genetically Engineered Crops: Experiences and Prospects*. Washington, DC: The National Academies Press.

National Family Farm Coalition et al. v Vilsack (2021) *Complaint for Declaratory & Equitable Relief Case No. 21-5695. The United States District Court for the Northern District of California*. Available at https://www.centerforfoodsafety.org/files/2021-07-26–ecf-01–nffc-et-al-complaint_33982.pdf.

Phillips, P. W. B. (2014). Economic Consequences of Regulations of GM Crops. Available at https://geneticliteracyproject.org/2014/12/11/economic-consequences-of-regulations-of-gm-crops/.

Plastina, A., Liu, F., Miguez, F., and Carlson, S. (2020). Cover Crops Use in Midwestern US Agriculture: Perceived Benefits and Net Returns. *Renew. Agric. Food Syst.* 35, 38–48. doi:10.1017/S1742170518000194

Qaim, M. (2020). Role of New Plant Breeding Technologies for Food Security and Sustainable Agricultural Development. *Appl. Econ. Perspect. Pol.* 42, 129–150. doi:10.1002/aepp.13044

Resnik, D. B. (2018). Ethics of Community Engagement in Field Trials of Genetically Modified Mosquitoes. *Developing World Bioeth.* 18, 135–143. doi:10.1111/dewb.12147

Roberts, P., Herkert, J., Kuzma, J., Iles, A., and Macnaghten, P. (2020). Responsible Innovation in Biotechnology: Stakeholder Attitudes and Implications for Research Policy. *Elementa-Sci Anthrop* 8, 47. doi:10.1525/elementa.446

Rockström, J., Edenhofer, O., Gaertner, J., and DeClerck, F. (2020). Planet-proofing the Global Food System. *Nat. Food* 1, 3–5.

Rose, K. M., Howell, E. L., Su, L. Y.-F., Xenos, M. A., Brossard, D., and Scheufele, D. A. (2019). Distinguishing Scientific Knowledge: The Impact of Different Measures of Knowledge on Genetically Modified Food Attitudes. *Public Underst Sci.* 28, 449–467. doi:10.1177/0963662518824837

Runck, B. C., Khoury, C. K., Ewing, P. M., and Kantar, M. (2020). The Hidden Land Use Cost of Upscaling Cover Crops. *Commun. Biol.* 3, 300–304. doi:10.1038/s42003-020-1022-1

Rundquist, S., and Cox, C. (2021). "In the Corn Belt, planting of cover crops plateaus, even as interest grows in their potential to address the climate crisis",in *Environmental Working Group*. Available at https://www.ewg.org/research/corn-belt-planting-cover-crops-plateaus-even-interest-grows-their-potential-address.

Runck, B. C., Kantar, M. B., Jordan, N. R., Anderson, J. A., Wyse, D. L., Eckberg, J. O., et al. (2014). The Reflective Plant Breeding Paradigm: A Robust System of Germplasm Development to Support Strategic Diversification of Agroecosystems. *Crop Sci.* 54, 1939–1948. doi:10.2135/cropsci2014.03.0195

Schiek, B., Hareau, G., Baguma, Y., Medakker, A., Douches, D., Shotkoski, F., et al. (2016). Demystification of GM Crop Costs: Releasing Late Blight Resistant Potato Varieties as Public Goods in Developing Countries. *Ijbt* 14, 112–131. doi:10.1504/ijbt.2016.077942

Schulte, L. A., Niemi, J., Helmers, M. J., Liebman, M., Arbuckle, J. G., James, D. E., et al. (2017). Prairie Strips Improve Biodiversity and the Delivery of Multiple Ecosystem Services from Corn-Soybean Croplands. *Proc. Natl. Acad. Sci. USA* 114, 11247–11252. doi:10.1073/pnas.1620229114

Shew, A. M., Nalley, L. L., Snell, H. A., Nayga, R. M., Jr, and Dixon, B. L. (2018). CRISPR versus GMOs: Public Acceptance and Valuation. *Glob. Food Security* 19, 71–80. doi:10.1016/j.gfs.2018.10.005

Siegrist, M., Connor, M., and Keller, C. (2012). Trust, Confidence, Procedural Fairness, Outcome Fairness, Moral Conviction, and the Acceptance of GM Field Experiments. *Risk Anal.* 32, 1394–1403. doi:10.1111/j.1539-6924.2011.01739.x

Siegrist, M. (2008). Factors Influencing Public Acceptance of Innovative Food Technologies and Products. *Trends Food Sci. Techn.* 19, 603–608. doi:10.1016/j.tifs.2008.01.017

Slovic, P. (1987). Perception of Risk. *Science* 236, 280–285. doi:10.1126/science.3563507

Steiner, A., Aguilar, G., Bomba, K., Bonilla, J. P., Campbell, A., Echeverria, R., et al. (2020). *Actions to Transform Food Systems under Climate Change. Wageningen, The Netherlands: CGIAR Research Program on Climate Change, Agriculture and Food Security (CCAFS)*.

Stokstad, E. (2020). United States Relaxes Rules for Biotech Crops. *Sci. May* 18, 2020. doi:10.1126/science.abc8305

Sturgis, P., and Allum, N. (2004). Science in Society: Re-evaluating the Deficit Model of Public Attitudes. *Public Underst Sci.* 13, 55–74. doi:10.1177/0963662504042690

Tamburini, G., Bommarco, R., Wanger, T. C., Kremen, C., van der Heijden, M. G. A., Liebman, M., et al. (2020). Agricultural Diversification Promotes Multiple Ecosystem Services without Compromising Yield. *Sci. Adv.* 6, eaba1715. doi:10.1126/sciadv.aba1715

The Center for Food Integrity (2020). "Coalition for Responsible Gene Editing in Agriculture" https://geneediting.foodintegrity.org/.

Thompson, P. B., Kassem, M., and Werner, W. G. (2007). *Food Biotechnology in Ethical Perspective*. Dordercht: Springer.

Tucker, J. J., III, and Jones, S. (2020). Environmental, Social, and Governance Investing: Investor Demand, the Great Wealth Transfer, and Strategies for ESG Investing. *J. Financ. Serv. Res.* 74.

USDA (2018). *Confirmation of the Regulatory Status of Thlapsi Arvense L. (Pennycress) Mutant Plant Lines*. https://www.aphis.usda.gov/biotechnology/downloads/reg_loi/18-337-01_air_response_signed.pdf.

USDA (2020). Database of Am I Regulated? Letters of Inquiry. Available at: https://www.aphis.usda.gov/aphis/ourfocus/biotechnology/am-i-regulated/regulated_article_letters_of_inquiry/regulated_article_letters_of_inquiry.

Wayman, S., Kissing Kucek, L., Mirsky, S. B., Ackroyd, V., Cordeau, S., and Ryan, M. R. (2017). Organic and Conventional Farmers Differ in Their Perspectives on Cover Crop Use and Breeding. *Renew. Agric. Food Syst.* 32, 376–385. doi:10.1017/S1742170516000338

Wigboldus, S., Klerkx, L., and Leeuwis, C. (2020). "Making Scale Work for Sustainable Development," in *Science, Technology, and Innovation for Sustainable Development Goals: Insights from Agriculture, Health, Environment, and Energy*. Editors A. A. Adenle, M. R. Chertow, E. H. Moors, and D. J. Pannell (Oxford University Press), 518–544. doi:10.1093/oso/9780190949501.003.0025

Willett, W., Rockström, J., Loken, B., Springmann, M., LangVermeulen, T. S., Garnett, T., et al. (2019). Food in the Anthropocene: the EAT-Lancet Commission on Healthy Diets from Sustainable Food Systems. *The Lancet* 393, 447–492. doi:10.1016/S0140-6736(18)31788-4

Yue, C., Shuoli, Z., Zhao, S., and Kuzma, J. (2015b). Heterogeneous Consumer Preferences for Nanotechnology and Genetic-Modification Technology in Food Products. *J. Agric. Econ.* 66, 308–328. doi:10.1111/1477-9552.12090

Yue, C., Zhao, S., Cummings, C., and Kuzma, J. (2015a). Investigating Factors Influencing Consumer Willingness to Buy GM Food and Nano-Food. *J. Nanopart Res.* 17, 283. doi:10.1007/s11051-015-3084-4

Zanetti, F., Alberghini, B., Jeromela, A. M., Grahovac, N., Rajković, D., Kiprovski, B., et al. (2021). Camelina, an Ancient Oilseed Crop Activel Contributing to the Rural Renaissance in Europe. A Review. *Agron. Sustain. Dev.* 41, 1–18. doi:10.1007/s13593-020-00663-y

Conflict of Interest: Authors KM and EW-L were employed by the company Terraluna Collaborative.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Bioengineering and Biotechnology

ORIGINAL RESEARCH
published: 02 May 2022
doi: 10.3389/fbioe.2022.886765

Lessons for a SECURE Future: Evaluating Diversity in Crop Biotechnology Across Regulatory Regimes

Dalton R. George[1,2], Eli D. Hornstein[3,2], Carrie A. Clower[4,2], Allison L. Coomber[5,2], DeShae Dillard[6,2], Nassib Mugwanya[7,2], Daniela T. Pezzini[6,2] and Casey Rozowski[2,8]*

[1]Department of Forestry and Environmental Resources, College of Natural Resources, North Carolina Sate University, Raleigh, NC, United States, [2]Genetic Engineering and Society Center, North Carolina State University, Raleigh, NC, United States, [3]Department of Plant and Microbial Biology, College of Agriculture and Life Sciences, North Carolina State University, Raleigh, NC, United States, [4]Department of Communication, College of Humanities and Social Sciences, North Carolina State University, Raleigh, NC, United States, [5]Department of Biological Sciences, College of Sciences, North Carolina State University, Raleigh, NC, United States, [6]Department of Entomology and Plant Pathology, College of Agriculture and Life Sciences, North Carolina State University, Raleigh, NC, United States, [7]Department of Agricultural and Human Sciences, College of Agriculture and Life Sciences, North Carolina State University, Raleigh, NC, United States, [8]Department of Agricultural and Resource Economics, College of Agriculture and Life Sciences, North Carolina State University, Raleigh, NC, United States

OPEN ACCESS

Edited by:
Carmen Vicien, University of Buenos Aires, Argentina

Reviewed by:
Umesh Jagtap, Rajaram College Kolhapur, India
Suresh Babu Naidu Krishna, Durban University of Technology, South Africa

***Correspondence:**
Dalton R. George
drgeorg2@ncsu.edu

Specialty section:
This article was submitted to Biosafety and Biosecurity, a section of the journal Frontiers in Bioengineering and Biotechnology

Received: *28 February 2022*
Accepted: *08 April 2022*
Published: *02 May 2022*

Citation:
George DR, Hornstein ED, Clower CA, Coomber AL, Dillard D, Mugwanya N, Pezzini DT and Rozowski C (2022) Lessons for a SECURE Future: Evaluating Diversity in Crop Biotechnology Across Regulatory Regimes. Front. Bioeng. Biotechnol. 10:886765. doi: 10.3389/fbioe.2022.886765

Regulation of next-generation crops in the United States under the newly implemented "SECURE" rule promises to diversify innovation in agricultural biotechnology. Specifically, SECURE promises to expand the number of products eligible for regulatory exemption, which proponents theorize will increase the variety of traits, genes, organisms, and developers involved in developing crop biotechnology. However, few data-driven studies have looked back at the history of crop biotechnology to understand how specific regulatory pathways have affected diversity in crop biotechnology and how those patterns might change over time. In this article, we draw upon 30 years of regulatory submission data to 1) understand historical diversification trends across the landscape and history of past crop biotechnology regulatory pathways and 2) forecast how the new SECURE regulations might affect future diversification trends. Our goal is to apply an empirical approach to exploring the relationship between regulation and diversity in crop biotechnology and provide a basis for future data-driven analysis of regulatory outcomes. Based on our analysis, we suggest that diversity in crop biotechnology does not follow a single trajectory dictated by the shifts in regulation, and outcomes of SECURE might be more varied and restrictive despite the revamped exemption categories. In addition, the concept of confidential business information and its relationship to past and future biotechnology regulation is reviewed in light of our analysis.

Keywords: crop biotechnology, SECURE rule, regulation, diversity trends, innovation, United States

INTRODUCTION

Regulation of next-generation crops in the United States promises to diversify innovation in agricultural biotechnology. Similar to how CRISPR-Cas and other gene editing methods have been lauded for driving diverse innovations in crop biotech development (Ahmar et al., 2020; Gupta & Shukla, 2017; Arora & Narula, 2017; Nasti & Voytas, 2021; Gao, 2021), the new USDA SECURE rule has been framed as enhancing the capacity to bring diverse innovations to market (Hoffman, 2021; Barrangou, 2020; USDA APHIS, 2020a). Specifically, some have argued that SECURE will expand the number of products eligible for exemption (Davies and Basher, 2020; Stokstad, 2020), which will increase opportunities for resource limited developers to commercialize their products and contribute to the variety of traits being developed through biotechnology (Hoffman, 2021). However, the longstanding relationship between regulation and the diversification of crop biotechnology development is not well understood empirically. Some suggest that regulation generally has an inhibitory effect on biotechnology development *via* barriers in regulatory costs and trade limitations (Smyth, 2020; Steinwand & Ronald, 2020). Others have argued that regulation can improve biotechnology development by reducing industrial uncertainty (Hansen, 2001) and enhancing product stewardship (Mbabzi et al., 2021).

Amidst these established theoretical perspectives, few data-driven studies have taken a concrete look at regulatory submissions to understand diversity in crop biotechnology and regulation. One such study, performed by Whelan et al. (2020), provides an example of how valuable this approach can be to understanding the role of regulation in diversification trends of the types of traits, organisms, and developers present in *Argentina*'s crop biotechnology sector. Surprisingly, no such study has yet been performed in the United States. In this article, we present our own data-driven approach that draws upon data from 30 years of regulatory submissions to investigate the relationship between diversity in crop biotechnology and regulation in the United States. Our analysis produces insights on what diversity in crop biotechnology looks like under two different historic regulatory pathways, how it has changed over time, and how the implementation of new regulatory rules might impact these trends.

Diversification and Regulation

The first objective of our study was to understand diversification trends across the landscape and history of past crop biotechnology regulatory pathways. Diversification of genetic engineering, for the purposes of our study, is defined as the breadth of organisms, genes, and traits that can be targeted in crop biotechnology development (Kumlehn et al., 2018), and the types of developers participating in commercialization. Our first step was to understand the relationship between regulatory mechanisms and proposed innovation in an empirical way. Toward this objective, we ask three questions:

1 What kinds of organisms, genes, traits, and developers have been subjected to past regulatory pathways?
2 How did the diversity within these categories change over time?
3 In what ways were the parameters of the relevant regulatory pathway(s) responsible for any diversification trend?

To answer these questions, we draw upon 3 decades of publicly available PDNS and AIR regulatory submissions. For the past 35 years of regulation, most products have been brought to market using the Coordinated Framework for the Regulation of Biotechnology (Federal Register, 2020). Under this framework, the main regulatory trigger for engineered plants was the integration of any sequences derived from plant pest organisms. This included many genes of interest for agronomically relevant traits, as well as *Agrobacterium* T-DNA sequences used as engineering tools (Hoffman, 2021). To bring a genetically engineered plant to market, developers were required to submit a "Petition for Determination of Nonregulated Status" (PDNS) through the USDA Animal and Plant Health Inspection Service (APHIS). Petitions required developers to submit extensive data to APHIS to perform a full science-based risk assessment before being granted permission to bring a product to market. Details of risk assessment outcomes were made publicly available, in addition to all relevant documents associated with PDNS submissions. However, in the late 2000s, developers began proposing and utilizing GE methods that removed plant pest sequences from the final engineered crop product (Wolt et al., 2016a). A lack of pest sequences did not trigger the requirement for a traditional science-based risk assessment, creating a gap between developer's next-generation products and regulatory scrutiny.

In response to this gap in regulation, USDA APHIS developed the "Am I Regulated" (AIR) consultation process. Unlike PDNS, AIR was an optional process designed for developers to use when they wanted to confirm whether or not their products were exempt from regulation by the USDA. Products could theoretically be brought to market without this regulatory consultation, but developers still made substantial use of this service. AIR letters of inquiry and responses from the USDA are publicly available, providing a record of exempt products and their associated characteristics (APHIS, 2022). However, specific details on traits, genes, methods, and organisms are not always fully available in AIR letters of inquiry as companies are given the option to claim confidential business information. This is not the case in the PDNS submissions, where all data and correspondence shared between developers and regulators are a matter of public record.

The Future as SECURE

The second objective of our study is to forecast how the new SECURE regulations might affect diversification trends in regulatory submissions and outcomes. The SECURE rule changes what types of GE crops are subject to review, which reconfigures what is eligible for regulatory exemptions. For example, plants with limited gene edits remain exempt from regulation, while those with multiple edits, multi-base-pair edits, and template-directed repair of edits are now subject to review (USDA APHIS, 2020b). Transgenic plants that recapitulate a previous combination of plant, trait, and mode of action are now exempt from regulation, regardless of whether they use methods

that involve plant pest sequences, while novel transgenics are subject to review regardless of whether or not they make use of plant pest sequences (USDA APHIS, 2020b).

The review process itself has also been changed significantly as a new step called "regulatory status review" (RSR) replaces the traditional PDNS process. All submissions that undergo RSR are subjected first to an evaluation, which can take up to 180 days. This first step is designed to determine if the genetic engineering event requires a full evaluation by USDA APHIS based on "a plausible pathway to increased plant pest risk" (USDA APHIS, 2020d). If APHIS determines that a plausible pathway exists, the results of the RSR would direct the developer to request that APHIS complete a full evaluation of all factors of concern related to potential increased plant pest risk. Non-regulated status is then determined from the results of this full evaluation, which APHIS estimates will take up to 15 months (USDA APHIS, 2020a).

SECURE has attracted attention from both opponents and proponents of GE crop regulation, and prognostication about how it will shape the future of agricultural biotechnology. APHIS frames the new SECURE rule as "reducing the regulatory burden for developers," (USDA APHIS, 2020b, pg 29790) because it creates expanded categories of exemptions and reforms the review process to proceed in an expedited fashion (in some cases). Some scholars have argued that this will promote diversification (Hoffman, 2021) and democratization (Barrangou, 2020) of crop biotechnology, in part because of a greater capacity to efficiently move products through regulatory review. However, authors have also pointed to problems with SECURE self-exemption rules and non-disclosure norms. Primarily, the self-exemption rule has been critiqued as formally departing from a science-based risk assessment (Jaffe, 2020; Kuzma and Greiger, 2020), a longstanding norm in biotechnology regulation. Potential ramifications of self-exemption and non-disclosure practices under SECURE also include other issues related to international trade compliance (Grossman, 2020), domestic disclosure laws (Jaffe, 2020), and eroding public trust due to a lack of transparency in crop biotech development (Kuzma and Greiger, 2020).

It's not clear from prior analyses how SECURE will affect the diversification of crop biotech, nor how these trends compare to what has happened under past regulatory regimes. As cited above, analyses of SECURE have applauded the revamping of safety standards to enhance commercialization (Barrangou, 2020; Hoffman, 2021) and critiqued some of the socioeconomic implications of these new rules (Kuzma and Greiger, 2020; Jaffe, 2020). However, discussions around the past and future of crop biotechnology regulation—especially around SECURE—are largely grounded in conceptual and theoretical analyses. Empirical contents of regulatory submissions are rarely, if ever, brought into analyses in a systematic fashion.

To establish a more concrete idea of how the previous exemption and review systems will compare to the new RSR landscape, we use our archival data to simulate product exemptions, expedited reviews, and full reviews under SECURE. Our goal is to analyze claims that SECURE will open up the landscape of agricultural biotechnology to a greater diversity of crops, traits, genes, and developers. In addition, there is no academic source providing an overview of what types of organisms, traits, genes, and developers have gone through regulation in the United States like there is for other countries, such as *Argentina* (Whelan et al., 2020). We suggest that now, at the point of transition to a new regime, is the ideal time to develop such a data resource for the United States. Doing so will allow the research community to perform more grounded analyses of past regulatory regimes and understand how future innovations are likely to fare under SECURE.

MATERIALS AND METHODS

Our data collection was driven by a content analysis on documents from two public sources: USDA APHIS petitions for determination of nonregulated status (PDNS) and "Am I Regulated?" inquiry letters (AIRs). Both PDNSs and AIRs are public documents made available *via* PDF copies of the original submissions and agency responses on the website of the USDA Biotechnology Regulatory Service. Information was manually extracted by reading through each submission and coded into an Excel spreadsheet according to a coding scheme devised by the researchers to capture various kinds of data from the archival documents (described below). Each document was read and initially coded by one investigator, with coding decisions reviewed by a second investigator for reasons of inter-coder reliability and accuracy. Outside literature and related regulatory filings were consulted for clarity when needed. Target organisms, engineering methods, traits conferred, and genes affected were collected for all entries where available. The nature of all entities submitting petitions was also reviewed and categorized after data collection was complete. Where data of a certain type was submitted for review but not publicly available, a value of "redacted" was recorded (see **Supplementary Appendix S1**).

Target organism data of two types was collected. First, the organism identity at the species level was recorded. Second, a crop category based on the USDA Agricultural Census categories was determined based on explicit inclusion in the category or similarity to existing members. We added categories for organisms not present in the USDA system: engineered bacteria and fungi were grouped as "microorganism," and timber crops were categorized under "forestry." An "Other" category contains submissions such as the five separate glowing plants sent to AIR. The organism categories used were (in alphabetical order): fiber, forage, forestry, fruit, grain, microorganism, nursery and flowers, oilseeds and other commodities, other, and vegetable.

Engineering method data of three types was collected. First, the genetic transformation method used to introduce transgenes (including editing constructs) was recorded. Transformation methods included *Agrobacterium*, biolistic, and several less common methods. Null sergeants of engineered organisms were also recorded. Second, the nature of the genetic engineering process with regard to the gene of interest's function in producing a trait was encoded as overexpression, silencing (including sense and antisense suppression and RNAi), and gene editing. Finally, where gene editing was used, the type of editing technology was recorded.

Trait data of two types was collected. First, "Trait" consisted of a ~1–3 word description of the intended trait as given in the regulatory submission. The description includes the targeted trait

and the direction of change from the wildtype, e.g., "reduced toxicity." In some cases, proximate and distal traits were reported, and these were encoded together separated by a "_," e.g., "altered ethylene synthesis_early flowering." Traits were recorded in separate columns and individual values of yes or no were recorded for each trait to allow for the presence of multiple traits in a single plant. The functional category of each trait was encoded as: agronomic; herbicide resistance; resistance to bacteria, fungi, viruses, or insects; product quality; or other.

Gene data of two types was also collected. First, each gene engineered in each submission was recorded. Second, the species of origin of the engineered gene was determined and recorded alongside the gene itself. To record genes engineered with different homologs or versions across different regulatory submissions, a column containing a generic acronym of the gene name was created, and the distinctive gene name and species of origin as given in each submission was entered as a datapoint in that column. For example, editing of specific polyphenol oxidases in potato (*Solanum tuberosum*) and apple (*Malus domestica*) was recorded as values of "St-Ppo5" and "Md-Ppo2" under the single column "PPO-1."

Developer type was determined as government, university, non-governmental organization, small to medium enterprise (SME), or large commercial enterprise (LCE). LCEs are defined as companies who employ 250 or more individuals, and SMEs are those companies who employ less than 250 individuals (OECD, 2021). When necessary, additional literature, regulatory filings, and media reports were consulted to differentiate between the two types of commercial entities.

In our analysis of the categories for species, developer, and trait, we determined the percentage of submissions falling under each crop category, entity type, and trait functional category, respectively. We also determined the average number of traits per submission and genes per trait by dividing the sum of all traits by the number of submissions and the sum of all genes by the sum of all traits, respectively, for each year in each process and plotted these against time. To enable direct comparison of submissions to PDNS and AIR, we controlled for differences in the timespan and total number of submissions between the two processes. First, we limited submissions to both processes to only those made from 2011 to 2020, the time period in which both AIR and PDNS were active. Then, we removed submissions where information in a given category was not disclosed due to a claim of confidential business information. Then, we normalized our diversity categories (total unique developers, organisms, traits, and genes) to the total number of submissions to each pathway in this time period. This gives an indication of how diverse submissions are to each pathway in terms of how often a new submission involves a data point that has not been seen before, independent of the total volume of submissions. These simple "diversity ratios" may be interpreted as: "on average, *n* unique data points are added to category X with every new submission." In addition to controlling for differences in submission rate and timeframe, the goal of using these ratios is to have a metric that reflects the relationship of the regulatory processes themselves to the broader biotech development system that generates submissions.

An important part of this study was to use the past regulatory submission data to forecast how the biotech regulatory landscape under the new SECURE rule would treat different product types. To do this, we analyzed how many of the 301 regulatory submissions from AIR and PDNS would have qualified for an exemption category or proceeded to RSR had SECURE been in place at the time of their development. For analysis of projected outcomes under the SECURE rule, we characterized each entry in our database as a predicted exemption when corresponding to a set of values, as specified in **Table 1** (see Methods Supplement). Outputs indicated which events in AIR and PDNS submissions would theoretically have been "regulated" and required an RSR or "exempted" based on a number of categorizations for novel products. Categorizations that would indicate an exempt product include: 1) if a product contains a single, non-template-guided gene edit, 2) a product is a null segregant of an engineered line with no remaining engineered genes, 3) a product contains an insertion of a single gene from the same species as the host organism or 4) a product is a repetition of a previously deregulated plant-trait-mode of action combination.

RESULTS

Overview of AIR and PDNS

When comparing an overview of all 301 submissions across the full time periods in which each process was active, it is notable that AIR attracted more total submissions (170) than PDNS(131) despite being active for a much shorter time. The AIR process appears more diverse in all aspects of diversity that we considered. In terms of developers (**Figure 1A**), AIR received submissions from 66 separate entities spread across all five developer categories. SMEs and universities were the largest contributors of AIR submissions with 86 and 46, respectively, followed by LCEs with 29 submissions. PDNS received submissions from 38 unique entities, dominated by 107 submissions from LCEs, followed by 21 from SMEs and a combined five submissions from government entities and universities.

The distribution of submissions across crop categories also differs with regulatory process and developer type (**Figure 1A**). Submissions by LCEs in the top two crop categories ("grains" and "oilseeds and other commodities") account for more than half of all submissions to PDNS. In contrast, AIR submissions are not dominated by submissions of any one crop type; no crop category makes up more than 50% of all submissions or more than 50% of the submissions from any developer category. To reach the simplest possible majority of AIR submissions, it is necessary to combine submissions across four crop categories and three developer categories.

Engineering methods also varied across AIR and PDNS (**Figure 1B**). As expected, the great majority of PDNS submissions involved expression of one or more transgenes. Gene editing was the most common genetic engineering event in AIR submissions, followed by transgene expression. In terms of the transformation method, the great majority of submissions to PDNS used *Agrobacterium*. No one transformation method accounted for a majority of AIR submissions, with a slim

TABLE 1 | Definitions of terms used in this article.

Term	Abbreviation	Definition
Am I Regulated? inquiry letters	AIR	A regulatory pathway where genetic engineering developers could submit a letter for review by APHIS to determine if their product was a regulated article. Discontinued in June 2020 and later replaced by SECURE.
Confidential business information		Competitive information pertaining to trade secrets, intellectual property, or other protected assets which are often redacted from AIR letters.
Diversity in biotechnology		The breadth of organisms, genes, and traits that can be targeted in genetic engineering development.
Genetically engineered	GE	The usage of biotechnology to alter or otherwise manipulate the genetic makeup of an organism.
Large Corporate Enterprise	LCE	A company which employs more than 250 individuals.
Petition for the Determination of Nonregulated Status	PDNS	A regulatory pathway where genetic engineering developers could submit a petition to APHIS to determine if a plant engineered with a plant pest posed a plant pest risk. Now replaced by SECURE.
Regulatory Status Review	RSR	A regulatory pathway where genetic engineering developers can request a review of a new genetically engineered plant which has not previously been given nonregulated status.
Small-to-Medium Enterprise	SME	A company which employs less than 250 individuals.
Sustainable, Ecological, Consistent, Uniform, Responsible, and Efficient ruling	SECURE	Revisions to APHIS's biotechnology regulations which reduce barriers to genetic engineering products which do not pose a plant pest risk. Became fully effective in Fall 2021.

plurality using biolistic gene delivery. A significant portion of AIR submissions did not disclose their transformation method or were not tied to a specific method; "undisclosed/unspecified" was the second most common classification after biolistic transformation. Notably, the transformation method varied significantly with the type of genetic engineering event: the great majority of gene-edited AIR submissions used *Agrobacterium* delivery, while the great majority of transgene-expression submissions used biolistics.

A greater number of total unique traits and genes was submitted to AIR than to PDNS (**Figure 2**). The difference in trait numbers was 85 total for the AIR submissions, and 45 for the PDNS, while for genes it was 85 for AIR and 79 for PDNS. *Bt* transgenics submitted to PDNS are a notable contributor to the fact that the number of genes is similar across regulatory systems despite the difference in the number of traits. Sixteen discrete *Bt* genes were recorded in our dataset but contributed to only five discretely categorized insect resistance traits. As shown in **Figure 1B** and **Figure 2**, the genes and/or traits in a significant portion of AIR submissions are redacted. Because redacted submissions may target a gene or trait that is the same as an existing submission, or may target multiple genes and traits, it is impossible to know the exact values underlying these categories.

Comparing Diversity in AIR and PDNS

While a useful overview, the utility of comparing the two regulatory processes using raw data like in **Figures 1**, **2** is limited by: AIR attracting a greater total number of submissions than PDNS, and PDNS (1991–2021) existing for nearly three times as long as AIR (2010–2021). Differences in the available technology at different times and the fact that AIR was not available as an alternative for most of the lifespan of PDNS could impact comparisons done with the raw data.

The diversity ratios for submissions from 2011 to 2020 (**Figure 3**) give a different view of the comparison. In addition to controlling for time, these values also control for the confounding factors mentioned above by normalizing to the total number of submissions (minus AIR submissions redacted for that category). As shown in **Figure 3**, in a direct comparison AIR no longer broadly leads PDNS. For Genes, the AIR pathway is still more diverse than PDNS. Notably, Genes have a diversity ratio greater than one in AIR, indicating the fact that on average more than one new gene was engineered for each new submission. The Developer diversity ratio is also higher for AIR (0.38) than for PDNS (0.3) showing that a new submission to AIR is more likely to come from a first-time developer. In Organisms, PDNS and AIR submissions are equal. PDNS submissions contained 0.36 new species per submission, while for AIR this was 0.35 species per submission. Notably, PDNS now clearly exceeds AIR in trait diversity, with developers submitting 0.81 new traits for every submission, while AIR submissions included 0.71 new traits each. Taken together, the results show that, after accounting for timespan and submission volume, the comparison of diversity in regulatory systems does indeed vary considerably across different facets of biotechnology. In some of these facets PDNS exceeds or equals AIR in its relationship to diversity.

The results in **Figure 3** also indicate that for several categories, the PDNS pathway must have had a greater level of diversity in the latter part of its existence from 2011 to 2020 than in the earlier years excluded from the time-corrected data in **Figure 3**. To further examine change over time in the PDNS process, we

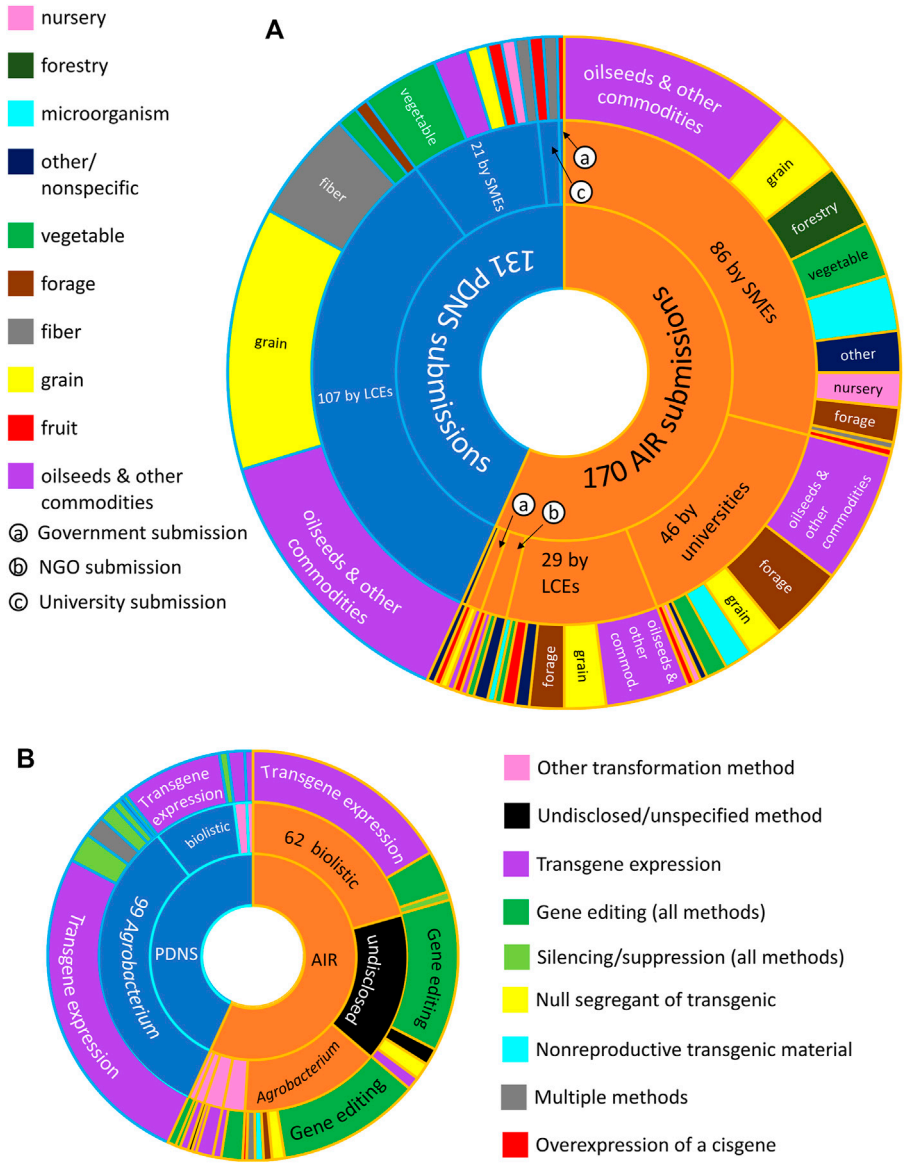


FIGURE 1 | Overview of different facts of diversity across AIR and PDNS. **(A)** Total submissions (innermost ring) and number of submissions contributed from each category of developer (center ring). The outermost ring shows the number of submissions in each crop category submitted by each category of developer. **(B)** Total submissions (innermost ring), transformation methods (center ring), and engineering methods (outermost ring) used in submissions to each regulatory pathway. Submissions containing undisclosed/redacted transformation methods (center ring) or engineering methods (outer ring) are shown in black.

compared the diversity ratios for all 30 years of PDNS submissions split into three 10-year periods.

Figure 4 demonstrates how diversity in submissions to PDNS has changed over time. For organisms and traits, diversity ratios have steadily increased over time from 1991–2020. For organisms, the ratios increase from 0.23, 0.31, to 0.36. For traits, the ratios increase from 0.37, to 0.5, to 0.81. Developer diversity remains relatively consistent at 0.35, 0.35, and 0.30, while gene ratios start at 0.5, increase to 0.81, but then drop to 0.68. The PDNS diversity over time shows that the timeframe of submissions–potentially reflecting differences in agricultural needs and/or the state of technology–alters the makeup of submissions, varies in its effect across diversity facets, and validates the need to control for it when comparing PDNS to AIR.

Traits, Crops, and Developers in Detail

To better understand what underlies the differences in different facets of diversity across submissions, we analyzed more detailed data within each category. Here, we show summary statistics and time-controlled comparisons of PDNS and AIR.

While **Figure 3** showed that over 2011–2020 AIR was slightly more diverse in total developers per submission than PDNS, **Figure 5** shows differences in the makeup of participants contributing to those figures. **Figure 5** shows the

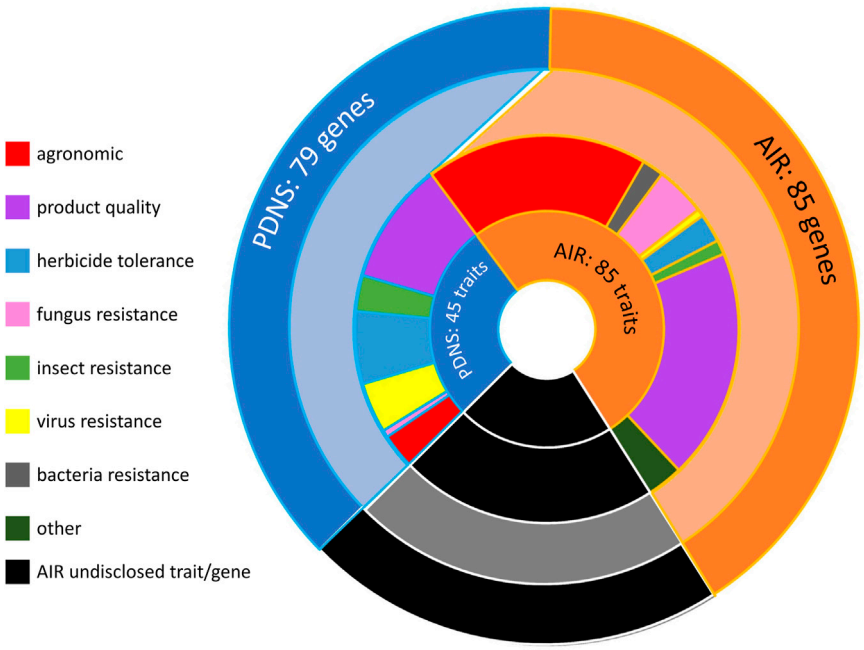


FIGURE 2 | The distribution of traits, trait categories, and total genes across all AIR and PDNS submissions. The innermost ring shows the number of unique traits engineered in submissions to each pathway. The next-innermost ring shows the number of unique traits engineered in each trait category across the two pathways, and the outermost ring shows the total number of unique genes engineered across all submissions to each pathway. AIR submissions in which the gene or trait of interest was redacted are represented in black. By their nature it is not possible to know the exact number of redacted genes or traits; this section of the graph is drawn proportionally to the number of submissions containing only redacted trait information.

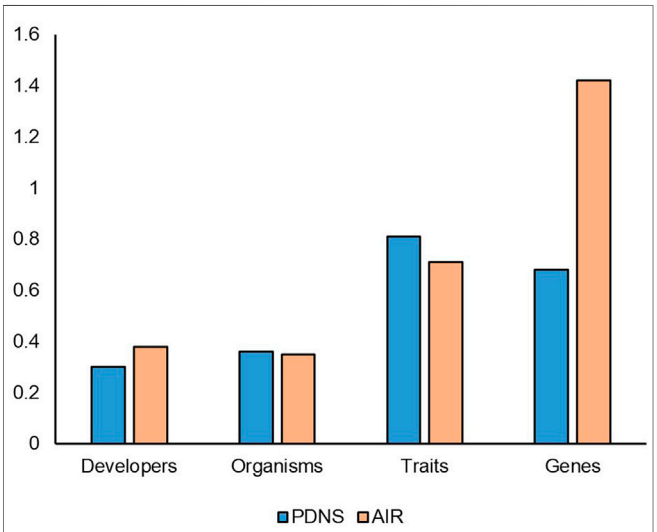


FIGURE 3 | Diversity ratios from 2011 to 2020. The number of submissions accounted for in this timeframe include all of the AIR submissions, but only the last 10 years of PDNS submissions. This timeframe reflects the years where the AIR & PDNS processes existed simultaneously. Redacted submissions in AIR are omitted from the ratio calculation.

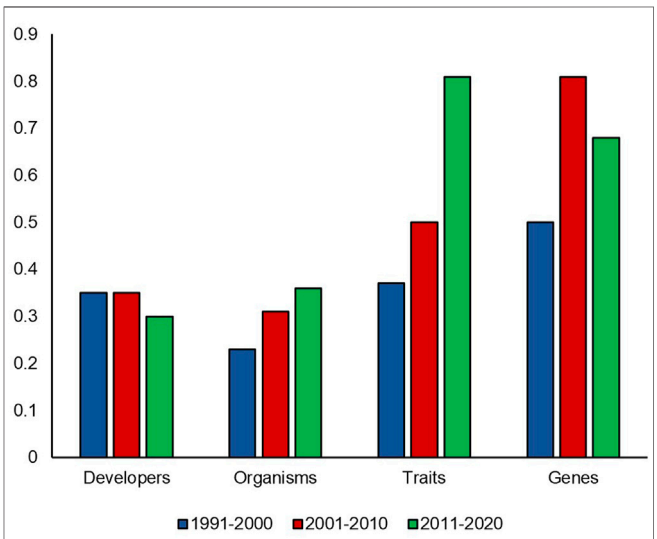


FIGURE 4 | PDNS diversity over time. Diversity ratios in each category were calculated for each 10 year timespan of the PDNS across all categories of interest.

percentage of submissions to AIR and PDNS from 2011 to 2020 contributed by each type of developer. Participants in AIR belonged to more categories, were more evenly distributed, and were dominated by different developer types than PDNS. 82% of submissions to PDNS were from LCEs. In contrast, the majority of submissions to AIR came from SMEs but this category did not as strongly dominate the

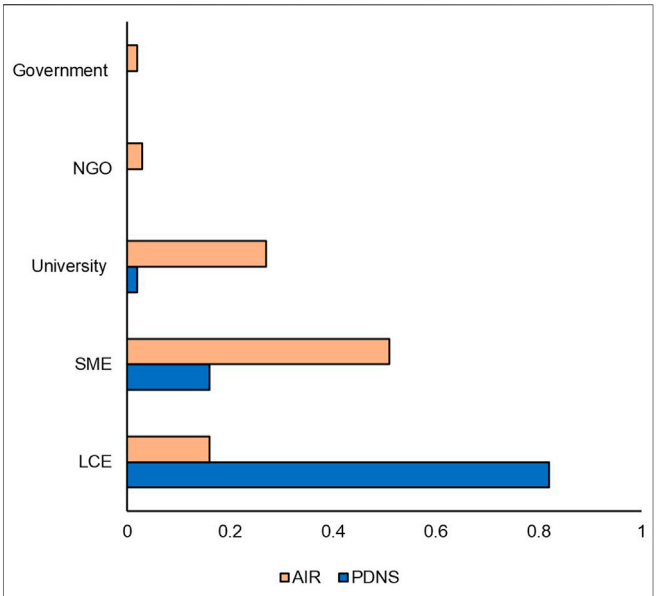


FIGURE 5 | Developer comparison. Percentages indicate the share of the total number of times that category occurred in the data set. Comparison is from 2011 to 2020.

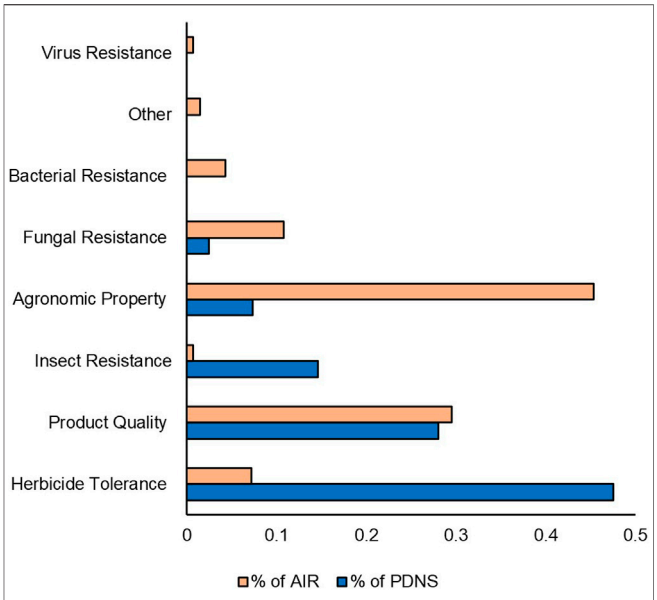


FIGURE 7 | Traits comparison. Traits were categorized using common USDA ERS designations. Percentages indicate the share of the total number of times that category occurred in the data set. Comparison is from 2011 to 2020.

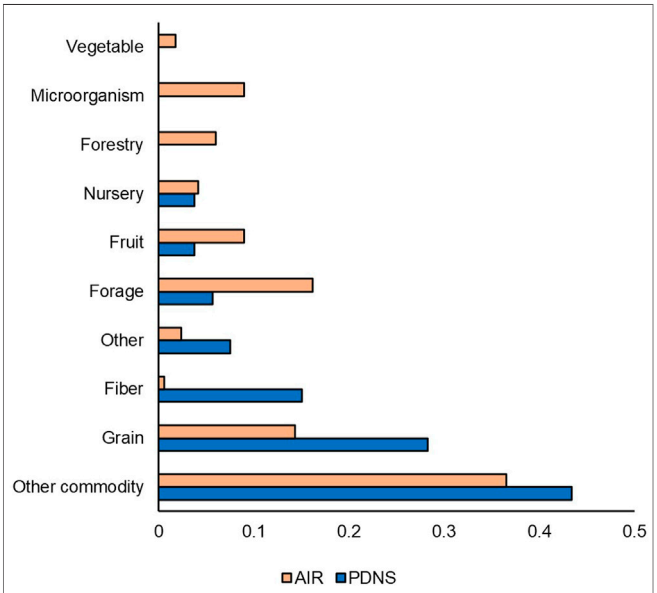


FIGURE 6 | Organism comparison. Unique organisms were categorized based on USDA Quick Stats & ERS crop designations, and researcher-assigned categories to cover organisms not addressed by USDA designations (i.e. microorganisms). Percentages indicate the share of the total number of times that category occurred in the data set. Comparison is from 2011 to 2020.

field, contributing 50% of submissions. The share of submissions to AIR from universities was also more than ten-fold greater than in PDNS, making universities the second-most prevalent contributor of AIR submissions.

Across all PDNS and AIR letters, our study registered a total of 81 unique organisms, with 62 coming from the AIR letters and 19 from the petitions. Only one species, chicory, appeared in PDNS but not AIR. In **Figure 6**, we compared the percentage of submissions to each regulatory process contributed by each of the crop categories from 2011 to 2020. AIR submissions were more diverse than PDNS, containing at least one submission to each of the ten categories. AIR submissions were also more evenly distributed: 87% of PDNS submissions but only 52% of AIRs came from the categories of 'Grain' 'Other Commodity,' and 'Fiber' which contain traditional row crops. A greater fraction of AIR submissions were devoted to the categories of Microorganism, Nursery, Fruit, Forestry, Forage, Other Commodity, and Other than in PDNS submissions.

Next, we compared the time-controlled dataset for differences in trait categories (**Figure 7**). **Figure 7** shows the percentage of submissions containing each trait category to AIR and PDNS from 2011 to 2020. Herbicide tolerance and insecticidal traits are much more prevalent in the PDNS data than AIR, making up 42.9% and 21.5%, as opposed to 7.2% and 1.4% respectively. In the AIR letters, agronomic properties were the most prevalent trait category, making up over 35% of the total reported trait targets. The trait category where both sets of articles were most similar was in product quality, which made up 29.5% of the AIR articles and 21.5% of the PDNS.

Lastly, our results determined how many distinct genes were engineered across both datasets. In total, 79 individual and unique genes were engineered in PDNSs and 85 genes were engineered in AIRs. We noted that the total unique genes number was significantly lower than the total submissions in both cases for different reasons. First, many PDNS submissions

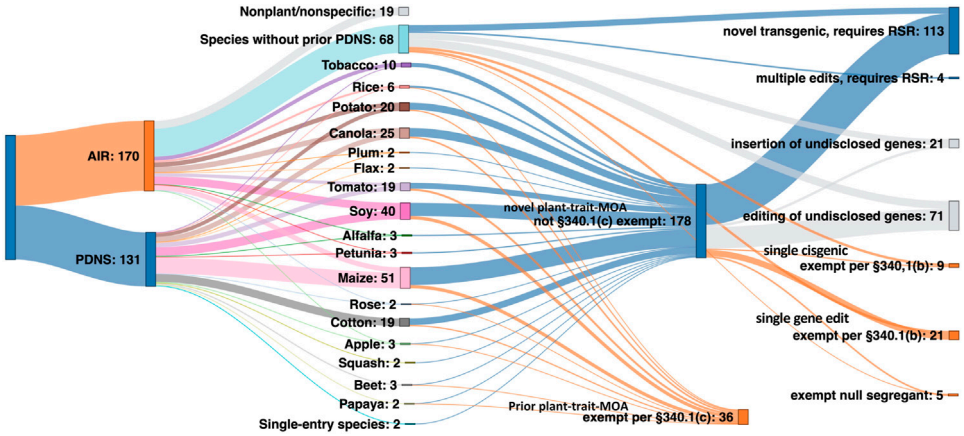


FIGURE 8 | The theoretical regulatory pathway of 30 years of US agricultural biotechnology submissions had they been considered under SECURE at the time of development. Starting from left: submissions under AIR and PDNS, the individual species they contain, and their projected exempt or regulated status under SECURE.

are focused on the same gene. Among PDNS the herbicide resistance genes *EPSPS* (glyphosate) and *Bar* (glufosinate) were the most commonly used, being inserted in 24 and 31 events respectively. We also noted that we treated 16 separate *Bt* toxins as individual genes in accord with their structure-function relationship and the approach taken by USDA in establishing Plant-Trait-MOA categories (USDA 2021). Second, in the AIRs, many submissions redacted the specific gene targeted. Among AIRs, 110 submissions did not disclose the identity of one or more genes. However, given the strong diversity ratio for the AIR gene category when using only submissions with identified genes (**Figure 3**), we expect that there are many more unique genes present in the products submitted to AIR.

Forecasting Future Regulations

An important part of this study was to use the past regulatory submission data to forecast how the biotech regulatory landscape under the new SECURE rule would treat different product types. To do this, we analyzed how many of the 301 regulatory submissions from AIR and PDNS would have qualified for an exemption category or proceeded to RSR (see **Supplementary Appendix S2**). **Figure 8** shows the projected outcomes of how submissions to AIR and PDNS might have been evaluated under SECURE.

Nineteen AIR submissions pertained to general technologies or to non-plant organisms, and were therefore excluded from the total analyzed submissions. Of the remaining 283 combined submissions, 41% (117 events) would clearly have proceeded to regulatory status review. 71 events, or 25% of the total, would have been exempt from review. The 340.1(c) exemption for new events of previous plant-trait and mode-of-action combinations submitted through the PDNS process contributed the majority of exemptions, with 340.1(b) exemptions for single gene editing events in AIR letters the next most prevalent.

The remaining 34% of all submissions contained too little information to establish with clarity whether an exemption would apply under SECURE, or if the submission would have proceeded to RSR. All of these submissions originated from AIR letters, and ambiguity was due to the level of redaction due to claimed confidential business information. Excluding all redacted AIR submissions reduces the total number of submissions in the analysis to 190. Out of this total, 59% (113) would be projected to require RSR under SECURE, while 41% (78) would have qualified for an exemption.

Interestingly, these results suggest a slightly higher number of crop biotechnological events would have been subject to at least the first stage of RSR than the PDNS pathway had SECURE been in place from 1991 to the present day. It is important to note that this result might underestimate the proportion of exemptions because it includes all PDNSs while excluding 2/3 of AIRs, which show a much higher ratio of exemptions. If, rather than being excluded, the redacted gene-edited and cisgenic AIRs are instead estimated to be exempt at the same rate as unredacted submissions in their respective categories, an additional 68 exempt and 24 nonexempt events are added. This reduces the total number of submissions projected to require RSR to 48% (137), and increases exemptions to 52% (146).

DISCUSSION

Our study conducted a data-driven examination of anecdotal observations on the regulation of crop biotech. Our aim was to evaluate claims about the relationship between regulation and diversity in crop biotech, and provide more carefully defined metrics that can be used in future work. Our results show that diversity in crop biotech does not follow a single trajectory dictated by shifts in regulation.

AIR and PDNS are very different pathways in terms of process, and at first glance it might appear that AIR submissions would be more diverse than the PDNS. Yet when we defined diversity as the breadth of unique traits, organisms, developers, and genes passing through a regulatory pathway per submission, and compared AIR and PDNS over the period of the past 10 years where both were

active, we found only slight differences in overall diversity (**Figure 3**) despite a large difference in the volume of submissions (**Figure 1**). Although AIR submissions contained more total members of each diversity category, most of this effect is explained by the fact that AIR simply attracted more submissions than PDNS. Combined, these results suggest that while AIR allowed inventions to more rapidly accumulate in the market, submissions directed to this regulatory pathway were not drastically more or less likely than in PDNS to involve a truly unique developer, organism, or trait.

AIR had a greater ratio of genes to submissions than PDNS. This is notable given that the same pattern is not seen in the ratio of traits to submissions (**Figure 3**), where PDNS slightly exceeds AIR. This suggests that AIR submissions engineered different genes to achieve the same trait, or are more likely to engineer multiple genes to achieve a trait. One obvious source of this effect is the inclusion of gene editing in only AIR data and its relationship to species. Each instance of editing the homolog of a gene in a new species was recorded as an additional engineered gene, while instances of inserting a previously used transgene into a new species were not considered to involve an additional engineered gene. It may also be the case that use of gene editing to achieve a trait genuinely requires targeting of a more diverse set of genes, or of additional genes, compared to transgenic methods. A more rigorous investigation of the genetic technology underlying these submissions, potentially accounting for incremental alterations to transgenes of interest over time and for the effect of regulatory and other noncoding sequences, may resolve the patterns that our preliminary results suggest are present.

We also observed that for organisms and traits, the PDNS submissions demonstrated a steady increase in diversity ratios over time (**Figure 4**). This is consistent with the fact that PDNS is more comparable to AIR in the timeframe of 2011–2020 (**Figure 3**) than over the full timeframe (**Figures 1**, **2**). This also suggests that, regardless of regulatory pathway, diversity in organisms and traits targeted by agricultural biotechnology and submitted to regulation was increasing. We hypothesize this is reflecting a continuous increase in expertise and application over time, which are not held static by the regulations in place at a given time. This is potentially a key point in gauging the impact of regulatory barriers on the overall progress of the field. Many contributors to advancement in biotechnology are academic and non-US based researchers whose work is not directly affected by US regulations that govern commercialization.

In contrast to the relative similarity of AIR and PDNS in the most broad measures of diversity, important nuances did appear in the specific types of developers, traits, organisms, and genes present across the different pathways. For developers, it was clear from our results that LCEs were much more likely to use the PDNS pathway, while SMEs and universities were much more likely to use AIRs. For traits, PDNS submissions showed a greater incidence of products aimed at insect resistance and herbicide tolerance, while the AIR letters were much more focused on agronomic properties (**Figure 7**). Lastly, for organisms, both PDNS and AIR were comparable for commodities, but varied across other crop categories like grain, forage, vegetable, and fiber crops (**Figure 6**).

These differences reflect an interesting feature of the interplay between the parallel biotechnology regulatory pathways that have been in place for the last decade. The LCEs that dominate transgenic submissions to PDNS did not abandon this work in favor of gene editing, nor did they assume a parallel dominant position in AIR submissions commensurate with their share of PDNS. This suggests that the draw of a lower regulatory barrier in AIR acts differently on different types of developers and, as has been previously suggested (Hoffman, 2021), does attract a significantly greater share of smaller developers who are not invested in the legacy traits of herbicide tolerance and insect resistance. Even though submissions to AIR are only slightly more likely to come from a new developer, they are much more likely to come from an SME or university.

To better understand how SECURE will alter regulation of biotechnology, we subjected the combined body of past PDNS and AIR submissions to simulated regulation. We determined their eligibility for various exemptions or requirements had they been submitted for review under SECURE, based on the technological characteristics we recorded. We found that while a significant number of submissions changed from exempt to regulated and vice versa, the end result was not a drastic shift in either direction. The real-world data included 131 products deregulated *via* PDNS, 165 confirmed exempt from regulation *via* AIR, and five found to be regulated products *via* AIR consultation. Our simulated regulation of these products under SECURE led to 113 regulated products, 71 exempt products, and 92 products with status we could not determine due to redacted technical information in the AIR letters. Even if all of the 92 uncategorized products, which mostly resulted from gene editing, are assumed to be exempt, this provides a final ratio of 40.9% regulated to 59.1% unregulated products in our theoretical SECURE regulation, as compared to 43.2% regulated and 56.8% unregulated products in the set of biotechnology products regulated through PDNS and AIR. Thus, while much of the discussion on SECURE has related to its regulatory exemptions, our results show that its impact when applied to the actual agricultural biotechnologies regulated in the US to date ranges from roughly equivalent to more restrictive than the prior PDNS/AIR parallel system in regard to exemptions.

Future Work on SECURE

Our investigation demonstrates that insights into the relationship between diversity and regulation can be garnered from studying official submissions to regulatory pathways, and that having transparent data is key to performing high quality analysis. Therein lies significant challenges for future work that might study the relationship between SECURE and crop biotechnology diversity. A surprising finding in the AIR letters was the high prevalence of confidential business information claims to avoid disclosing genes, methods, and traits, sometimes all in the same submission. At least one redaction from one of these categories was found in over half of AIR letters. We suggest that this indicates an overarching interest in privacy on the part of biotechnology developers.

The ability to withhold information that impacts competition and intellectual property is a potentially overlooked factor in discussions about what attracts developers to a regulatory

pathway, and may be overshadowed by discussions on regulatory review processes and the barriers they empose. Discussion on the biotechnology regulation-innovation relationship has focused on the burden in terms of costs, time, and data. Looking forward, we should consider the indirect effect of enforcing disclosures on regulatory choices. Protecting competitive information may be as or more important than other costs when biotechnology developers choose to orient their technology towards a particular regulatory path.

An interesting aspect of confidential business information redaction in past regulation is that it was, in theory, permitted in PDNSs under the same set of justifications used for AIRs which specifies "genotypes, phenotypes, donor organisms, gene names, gene description, and transformation method" as prospective confidential business information (USDA APHIS, 2020c7 CFR 340.6). The greater degree of disclosure in PDNSs may be a product of administrative decision making on the part of USDA, strategic choices by developers, or simply a tendency to follow the example of earlier submissions. SECURE's RSR also operates under these general rules, and an essential component of the future under SECURE will be whether RSR disclosures tend to treat confidential business information more similarly to AIR letters or PDNSs. If RSRs permit greater confidentiality than PDNSs, this will provide a means of comparing the relative importance of regulatory burden and confidentiality in shaping biotech developers' incorporation of regulatory effects into their technology development decisions.

Regardless, it is unlikely that SECURE will actively increase the extent of disclosures made by biotech developers relative to past regimes, and it certainly does not mandate this practice. We may therefore experience a hidden diversity in crop biotech development, where many different novel products enter the market, but without the public being notified in any meaningful way (Kuzma and Greiger, 2020). This will make future evaluation of agricultural biotechnology difficult, and may degrade public trust due to a lack of transparency and engagement (Kuzma, 2018). The use of exemptions and truncated reviews also raises questions concerning accountability, and what happens in the case of mistakes and unintended consequences. All stages of SECURE are oriented towards evaluating the trait and engineering method of interest as they are described to the USDA. However, skipping extensive regulatory review also reduces the opportunities for crop developers to confirm that the genetic makeup of their product is exactly as intended.

For developers taking advantage of exemptions and first-stage RSR, this is potentially a high-stakes proposition. An "invalid determination," e.g. misidentifying a known genetic change as exempt or releasing a product with additional unintended genetic changes that alter its regulatory category, can result in enforcement actions by APHIS including fines up to $1,000,000 and seizure of materials, as well as potential liability (USDA APHIS, 2020c 7 CFR 340.6, USDA APHIS, 2020b). These instances are not uncommon in standard plant engineering methods, and detection of off-target editing and silent gene insertions is frequently more difficult than engineering the trait of interest (GelvinGelvin 2003; Wolt et al., 2016b;2017; Zhang et al., 2018). How enforcement will work in practice is yet to be seen, especially in cases where some form of regulatory review for events later found to be misidentified has been undertaken by the government. However, pursuit of regulatory reassurance by choosing to submit to more extensive regulation than the minimum required can slow developers' path to commercialization and potentially impact their competitiveness. This may be especially important for smaller developers that have fewer resources and that, according to our data (**Figures 1**, **5**), are more likely to use exempt engineering methods.

We suggest that there is a combined solution to the problems of encouraging transparency, avoiding unintended engineering events, and enabling a diversity of engineering applications to be pursued by smaller developers and nonprofits. Kuzma and Grieger (2020) writing on this topic proposed a novel, voluntary, non-governmental system in which developers are incentivized to disclose basic details about their products in exchange for a certification that would "signify that the biotech crop producer is striving to become more transparent and trustworthy according to community-derived standards" (pg 917). This system, termed "CLEAR-GOV", would exist as a nonprofit staffed by experts in the field, and use the information contributed by developers in exchange for certification to construct a database for future academic work, public availability, and engagement (Kuzma and Greiger, 2020). We support this approach, and note that disclosure is not only a matter of transparency and building public trust, but could also be very beneficial to biotechnology developers' practical ability to operate under SECURE.

Our results suggest that a strong interest in privacy on the part of biotech developers leads them to often opt against transparency when given the choice in regulation. Participation in a voluntary transparency-focused initiative may therefore require an inducement that goes beyond a certification. The same body of developers opting for secrecy in past AIRs is also skewed towards less experienced and smaller entities. These developers benefit materially from understanding their own new technology's regulatory position and real-world utility, which will be much easier if they have access in uniform fashion to technical parameters of decisions and review undertaken by USDA that go beyond the minimum required by law. A transparent reporting system such as CLEAR-GOV could therefore be built on the additional strength of providing information that is valuable to developers themselves in pursuit of novel innovations, while simultaneously serving the ends of good governance.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: Petitions for Determination of non-regulated status (USDA APHIS) https://www.aphis.usda.gov/aphis/ourfocus/biotechnology/permits-notifications-petitions/petitions/petition-status Am I Regulated Letters of Inquiry (USDA APHIS) https://www.aphis.usda.gov/aphis/ourfocus/biotechnology/am-i-regulated.

AUTHOR CONTRIBUTIONS

All authors contributed to conception and design of the study. DG, EH, and NM organized the database. DG and EH performed the analysis. DG and EH wrote the first draft of the manuscript.

CC, AC, DD, and DP contributed to sections of the manuscript. All authors contributed to manuscript revision, read, and approved submitted version.

FUNDING

All authors were funded by the National Science Foundation NRT-INFEWS: Agricultural Biotechnology in our Evolving Food, Energy andWater Systems (#1828820) during this research. NSF provided funding for author fellowships administered by North Carolina State University's Genetic Engineering and Society Center.

ACKNOWLEDGMENTS

Special thanks to Dr. Jennifer Kuzma, Dr. Zachary Brown, & Dr. Fred Gould for providing helpful review and suggestions on the development of this manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2022.886765/full#supplementary-material

REFERENCES

Ahmar, S., Saeed, S., Khan, M. H. U., Ullah Khan, S., Mora-Poblete, F., Kamran, M., et al. (2020). A Revolution toward Gene-Editing Technology and its Application to Crop Improvement. *Ijms* 21 (16), 5665. doi:10.3390/ijms21165665

Arora, L., and Narula, A. (2017). Gene Editing and Crop Improvement Using CRISPR-Cas9 System. *Front. Plant Sci.* 8, 1932. doi:10.3389/fpls.2017.01932

Barrangou, R. (2020). Finding SECURE Ground: USDA Edits the Biotechnology Regulatory Framework. *CRISPR J.* 3 (3), 136–137. doi:10.1089/crispr.2020.29096.rba

Davies, S., and Brasher, P. (2020). USDA Eases Biotech Regulations to Exempt Some Crops. Available at: https://www.agri-pulse.com/articles/13694-usda-announces-regulatory-exemptions-for-ge-plants (Accessed: February 20, 2022).

Federal Register (2020). *Movement of Organisms Modified or Produced through Genetic Engineering, 7. CFR 340.*

Gao, C. (2021). Genome Engineering for Crop Improvement and Future Agriculture. *Cell* 184 (6), 1621–1635. doi:10.1016/j.cell.2021.01.005

Gelvin, S. B. (2003). Agrobacterium -Mediated Plant Transformation: the Biology behind the "Gene-Jockeying" Tool. *Microbiol. Mol. Biol. Rev.* 67, 16–37. doi:10.1128/MMBR.67.1.16-37.2003

Gelvin, S. B. (2017). Integration of Agrobacterium T-DNA into the Plant Genome. *Annu. Rev. Genet.* 51 (1), 195–217. doi:10.1146/annurev-genet-120215-035320

Grossman, M. R. (2020). The SECURE Rule: New Regulations for Crop Biotechnology in the United States. *Eur. Food Feed L. Rev. : EFFL* 15 (6), 548–562.

Gupta, S. K., and Shukla, P. (2017). Gene Editing for Cell Engineering: Trends and Applications. *Crit. Rev. Biotechnol.* 37 (5), 672–684. doi:10.1080/07388551.2016.1214557

Hansen, A. (2001). Biotechnology Regulation: Limiting or Contributing to Biotech Development? *New Genet. Soc.* 20 (3), 255–271. doi:10.1080/14636770120093010

Hoffman, N. E. (2021). Revisions to USDA Biotechnology Regulations: The SECURE Rule. *Proc. Natl. Acad. Sci. U.S.A.* 118 (22). doi:10.1073/pnas.2004841118

Jaffe, G. (2020). 'Ramifications of Exemption and Self-Determination Provisions in USDA's New SECURE Rule', Center for Science in the Public Interest. Available at: https://www.cspinet.org/news/biotech-blog-ramifications-exemption-and-self-determination-provisions-usda%E2%80%99s-new-secure-rule (Accessed: February 20, 2022).

Kumlehn, J., Pietralla, J., Hensel, G., Pacher, M., and Puchta, H. (2018). The CRISPR/Cas Revolution Continues: From Efficient Gene Editing for Crop Breeding to Plant Synthetic Biology. *J. Integr. Plant Biol.* 60 (12), 1127–1153. doi:10.1111/jipb.12734

Kuzma, J. (2018). *'Regulating Gene-Edited Crops', Issues Sci. Technology* 35 (1), 80–85.

Kuzma, J., and Grieger, K. (2020). Community-led Governance for Gene-Edited Crops. *Science* 370 (6519), 916–918. doi:10.1126/science.abd1512

Mbabazi, R., Koch, M., Maredia, K., and Guenthner, J. (2021). Crop Biotechnology and Product Stewardship. *GM Crops & Food* 12 (1), 106–114. doi:10.1080/21645698.2020.1822133

Nasti, R. A., and Voytas, D. F. (2021). Attaining the Promise of Plant Gene Editing at Scale. *Proc. Natl. Acad. Sci. U.S.A.* 118 (22). doi:10.1073/pnas.2004846117

Smyth, S. J. (2020). Regulatory Barriers to Improving Global Food Security. *Glob. Food Security* 26, 100440. doi:10.1016/j.gfs.2020.100440

Steinwand, M. A., and Ronald, P. C. (2020). Crop Biotechnology and the Future of Food. *Nat. Food* 1 (5), 273–283. doi:10.1038/s43016-020-0072-3

Stokstad, E. (2020). United States Relaxes Rules for Biotech Crops. Available at: https://www.science.org/content/article/united-states-relaxes-rules-biotech-crops (Accessed: February 20, 2022).doi:10.1126/science.abc8305

USDA APHIS (2020b). Am I Regulated" Process. Available at: https://www.aphis.usda.gov/aphis/ourfocus/biotechnology/am-i-regulated/am_i_regulated (Accessed: February 26, 2022).

USDA APHIS (2020d). Guidance for Requesting a Confirmation of Exemption from Regulation under 7 CFR Part 340. Available at: https://www.aphis.usda.gov/brs/pdf/requesting-confirmation-of-exemption.pdf (Accessed: February 26, 2022).

USDA APHIS (2020a). Movement of Certain Genetically Engineered Organisms. *Fed. Regist.* 85 (96).

USDA APHIS (2020c). *Petition for Determination of Non-regulated Status, 7 CFR 340*, 6.

Whelan, A. I., Gutti, P., and Lema, M. A. (2020). Gene Editing Regulation and Innovation Economics. *Front. Bioeng. Biotechnol.* 8, 303. doi:10.3389/fbioe.2020.00303

Wolt, J. D., Wang, K., Sashital, D., and Lawrence-Dill, C. J. (2016a). Achieving Plant CRISPR Targeting that Limits Off-Target Effects. *Plant Genome* 9 (3). doi:10.3835/plantgenome2016.05.0047

Wolt, J. D., Wang, K., and Yang, B. (2016b). The Regulatory Status of Genome-edited Crops. *Plant Biotechnol. J.* 14 (2), 510–518. doi:10.1111/pbi.12444

Zhang, Q., Xing, H.-L., Wang, Z.-P., Zhang, H.-Y., Yang, F., Wang, X.-C., et al. (20182018). Potential High-Frequency Off-Target Mutagenesis Induced by CRISPR/Cas9 in Arabidopsis and its Prevention. *Plant Mol. Biol.* 96, 445–456. doi:10.1007/s11103-018-0709-x

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Bioengineering and Biotechnology

REVIEW
published: 28 June 2022
doi: 10.3389/fbioe.2022.913728

CRISPR/Cas- and Topical RNAi-Based Technologies for Crop Management and Improvement: Reviewing the Risk Assessment and Challenges Towards a More Sustainable Agriculture

Fabiano Touzdjian Pinheiro Kohlrausch Távora[1,2], Francisco de Assis dos Santos Diniz[1], Camila de Moraes Rêgo-Machado[1,2], Natália Chagas Freitas[2], Fabrício Barbosa Monteiro Arraes[3], Eduardo Chumbinho de Andrade[4], Leila Lourenço Furtado[1], Karen Ofuji Osiro[1,2], Natália Lima de Sousa[5], Thiago Bérgamo Cardoso[6], Liliane Márcia Mertz Henning[7], Patrícia Abrão de Oliveira Molinari[2], Sérgio Enrique Feingold[5], Wayne B. Hunter[8], Maria Fátima Grossi de Sá[3], Adilson Kenji Kobayashi[2], Alexandre Lima Nepomuceno[7], Thaís Ribeiro Santiago[1] and Hugo Bruno Correa Molinari[6]**

[1]Department of Phytopathology, University of Brasília, Brasília, Brazil, [2]Embrapa Agroenergy, Brasília, Brazil, [3]Embrapa Genetic Resources and Biotechnology, Brasília, Brazil, [4]Embrapa Cassava and Fruits, Cruz Das Almas, Brazil, [5]IPADS-Balcarce (UEDD INTA-CONICET), Balcarce, Argentina, [6]SEMPRE AgTech, Chapecó, Brazil, [7]Embrapa Soybean, Londrina, Brazil, [8]USDA-ARS, U.S. Horticultural Research Laboratory, Fort Pierce, FL, United States

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated gene (Cas) system and RNA interference (RNAi)-based non-transgenic approaches are powerful technologies capable of revolutionizing plant research and breeding. In recent years, the use of these modern technologies has been explored in various sectors of agriculture, introducing or improving important agronomic traits in plant crops, such as increased yield, nutritional quality, abiotic- and, mostly, biotic-stress resistance. However, the limitations of each technique, public perception, and regulatory aspects are hindering its wide adoption for the development of new crop varieties or products. In an attempt to reverse these mishaps, scientists have been researching alternatives to increase the specificity, uptake, and stability of the CRISPR and RNAi system components in the target organism, as well as to reduce the chance of toxicity in nontarget organisms to minimize environmental risk, health problems, and regulatory issues. In this review, we discuss several aspects related to risk assessment, toxicity, and advances in the use of CRISPR/Cas and topical RNAi-based technologies in crop management and breeding. The present study also highlights the advantages and possible drawbacks of each technology, provides a brief overview of how to circumvent the off-target occurrence, the strategies to increase on-target specificity, the harm/benefits of association with nanotechnology, the public perception of the available techniques, worldwide regulatory frameworks regarding topical RNAi and CRISPR technologies, and, lastly, presents successful case studies of

OPEN ACCESS

Edited by:
Carmen Vicien,
University of Buenos Aires, Argentina

Reviewed by:
Gabriela Levitus,
ArgenBio, Argentina
Muhammad Sohaib Shafique,
Chinese Academy of Agricultural Sciences (CAAS), China

***Correspondence:**
Thaís Ribeiro Santiago
thais.santiago@unb.br
Hugo Bruno Correa Molinari
hm@sempre.agr.br

Specialty section:
This article was submitted to Biosafety and Biosecurity, a section of the journal Frontiers in Bioengineering and Biotechnology

Received: 06 April 2022
Accepted: 06 June 2022
Published: 28 June 2022

Citation:
Touzdjian Pinheiro Kohlrausch Távora F, de Assis dos Santos Diniz F, de Moraes Rêgo-Machado C, Chagas Freitas N, Barbosa Monteiro Arraes F, Chumbinho de Andrade E, Furtado LL, Osiro KO, Lima de Sousa N, Cardoso TB, Márcia Mertz Henning L, Abrão de Oliveira Molinari P, Feingold SE, Hunter WB, Fátima Grossi de Sá M, Kobayashi AK, Lima Nepomuceno A, Santiago TR and Correa Molinari HB (2022) CRISPR/Cas- and Topical RNAi-Based Technologies for Crop Management and Improvement: Reviewing the Risk Assessment and Challenges Towards a More Sustainable Agriculture. Front. Bioeng. Biotechnol. 10:913728. doi: 10.3389/fbioe.2022.913728

biotechnological solutions derived from both technologies, raising potential challenges to reach the market and being social and environmentally safe.

Keywords: exogenous dsRNA, genome editing, gene silencing, nanotechnology, offtargets, public acceptance, regulatory aspects, toxicity

1 AN OVERVIEW OF PLANT BREEDING: FROM ANCIENT TIMES TO GENETIC MANIPULATION ASSOCIATED WITH MOLECULAR BREEDING

The use of improved genotypes in agriculture started 10,000 years ago with the process of crop domestication when humans began to adapt wild plant species for cultivation as food plants (Doebley et al., 2006). For many years, conventional plant breeding has been performed by artificial crossing or induced random mutagenesis, and the selection of parents and descendants is based majorly on the phenotype, hence in the absence of molecular and physiological basis of enhanced traits (Jorasch, 2019). These breeding approaches, although time-consuming, labor-intensive, and randomly oriented to some extent, continue to deliver crop varieties supporting demands for increased agricultural production (Scheben et al., 2017).

In the last 30 years, biotechnology tools have allowed the development of desirable genotypes in less time and generally at a lower cost compared to conventional breeding. Modern agriculture has profited from advances in molecular biology and next-generation sequencing (NGS) technologies for high throughput sequencing, which revolutionized genetic plant breeding, with emphasis on transgenic technology, molecular markers, and genomic selection (Kim et al., 2020; Thudi et al., 2021).

Transgenic breeding has been the most frequent technique applied for plant genetic manipulation in history, allowing desirable target genes to be introduced into the plant genome ideally without making other unintended genetic changes (Qaim, 2020). These early developments showed the capability of genetically engineering a plant genome and inspired other breeding approaches such as gene silencing. The first report of gene silencing in plants was demonstrated in 1989 in tobacco plants (Matzke et al., 1989), and a subsequent study showed that the integration of transgenes homologous to plant endogenous genes could result in suppression of both expressed genes, a process called co-suppression (Napoli et al., 1990). Later, Fire et al. (Fire et al., 1998) used the nematode *Caenorhabditis elegans* to show for the first time that the suppression of target transcripts expression is triggered by double-stranded RNA (dsRNA) molecules, a mechanism known as "RNA interference" (RNAi). Since then, several components of the RNAi pathway were identified, and the practical use of RNAi-based GMOs has advanced rapidly (Saurabh et al., 2014). However, unlike GMO plants that are generally modified to express a specific protein, RNAi-based GMO plants have been modified to express dsRNA molecules that enable specific silencing of target genes on the plant or pathogen/pest genomes (Arpaia et al., 2020), a strategy termed host-induced gene silencing (HIGS). According to Ghag et al. (Ghag, 2017), HIGS was an innovative concept of RNAi technology for effective silencing of one or a few genes with agronomic importance. This technology has many potential applications in agriculture, including enhancing resistance against biotic and abiotic stresses, improving industrial and nutritional quality, delayed ripening, male sterility, plant architecture modification, and removal of allergens and toxins (Rajam, 2020).

RNAi pathways are natural mechanisms present in almost all eukaryotic organisms. Basically, these pathways work through processing long dsRNA into called small interfering RNA (siRNA) or micro-RNA (miRNA) molecules, which are responsible for recognizing the target messenger RNA (mRNA), as well as guiding the DNA and histone modifications or chromatin remodeling, leading to target gene silencing (Wilson and Doudna, 2013). RNAi-based GMOs have become key elements for plant breeding, due to their ability to modulate gene expression in a sequence-specific manner. However, there are great constraints and delicate issues related to the use of transgenics—including the transgenic approach of RNAi-based technology—that have negatively impacted the development of new GMO crops, such as high costs, negative perception of some consumers, long timelines to succeed, restrictive regulatory framework, and the lack of genetic transformation protocols for many crop species (Scheben et al., 2017; Fletcher et al., 2020). Despite the mentioned rapidness of the transgenic approach, the approval of a new GM plant takes, on average, 10–12 years of successive biochemical, molecular, environmental, and animal health-related trials, according to the regulation adopted by each country (Qaim, 2020).

In this context, since the early 2000s, the use of RNAi-based non-transgenic approaches (e.g., exogenous and self-deliverable dsRNA molecules) has been explored in agriculture, mostly for plant protection against pathogens and pests (Tenllado et al., 2003; Rego-Machado et al., 2020; Kalyandurg et al., 2021). This strategy, currently known as spray-induced gene silencing (SIGS), has been attempted as a potential and alternative biotechnological tool for transgenic plants, due to its appealing features, being too much faster, cheaper, easier to handle, and capable to encompass a broader range of target organisms (Rank and Koch, 2021), while avoiding plant transformation/screening steps, and biosafety issues in some extent. Furthermore, this approach holds enormous potential to meet the increasing public demand for reducing agrochemical applications toward more sustainable and agroecological production. In addition, SIGS has been shown to be more efficient under lab conditions compared to the HIGS strategy (Koch et al., 2016). Nowadays, there is mounting evidence suggesting that topically-applied dsRNAs molecules are effective in silencing target genes aiming at plant resistance against a broad range of biotic factors (Dubrovina and Kiselev, 2019; Dalakouras et al., 2020; Das and Sherif, 2020).

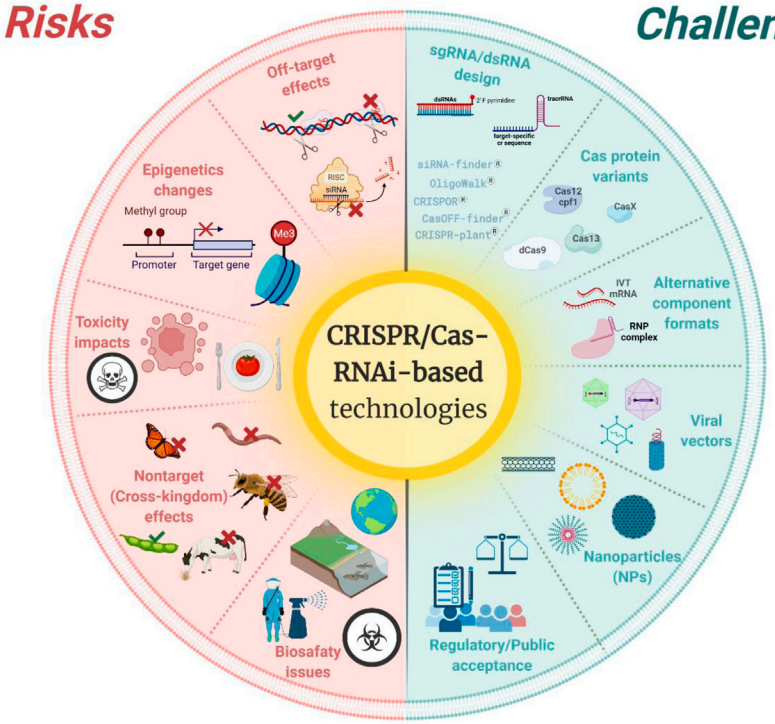


FIGURE 1 | Summary of the main risks (on the left—in red color) and challenges (on the right—in green color) addressed in the text and related to the application of Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated gene (Cas)- and RNA interference (RNAi)-based technologies in agriculture. Created with Biorender software at https://biorender.com.

Additionally, advances in genomics studies with nuclease enzymes have allowed the emergence of equally revolutionary novel non-transgenic tools that are used in site-directed genome editing for precision plant breeding, also not necessarily involving the integration of exogenous sequences into the plant genome. Based on the mode of action of these gene-editing tools, DNA is modified, inserted, replaced, or deleted in the plant genome at specific locations using sequence-specific nucleases, leading to gene modification at target sites. These genomic editing tools can be used to improve multiple traits simultaneously, controlled by multiple loci of the genome (Rodríguez-Leal et al., 2017), facilitating the development of commercial products, which is often difficult using conventional genetic breeding techniques.

In this decade, the most widely used gene-editing technology is the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated gene (Cas) system, an adaptive immunity mechanism found in bacteria and archaea against bacteriophages and mobile genetic elements, which was transformed as a genome editing biotechnological tool in 2012 (Jinek et al., 2012). This tool relies on a special site of the bacterial genome called CRISPR locus, which is a gene array composed of spacers acquired from the invader's exogenous DNA and integrated between small bacterial palindromic repeats. Flanking the CRISPR locus there are genes encoding Cas nucleases, responsible for cleavage of exogenous DNA upon new infection by the same invader. The spacers are transcribed into small guide RNAs that once complexed with Cas nucleases direct the breakdown of the intruder DNA (Marraffini, 2015). The use of CRISPR/Cas in plant breeding allows the segregation of system components (e.g., Cas protein and guide RNA—gRNA) out of the host genome, post-target gene editing, enabling the generation of non-transgenic crops. Moreover, for this purpose, the system has been experimentally optimized, and a transgene-free approach to the technology can be performed which usually involves the use of a ribonucleoprotein (RNP) complex made only by the gRNA and Cas nuclease protein transcribed *in vitro* (Zhang et al., 2021a).

Indeed, both technologies—RNAi and CRISPR/Cas—have the power to revolutionize plant research and breeding (Younis et al., 2014; Guo et al., 2016; Ricroch and Hénard-Damave, 2016). In this review, we present an up-to-date panorama on advancements and breakthroughs of both technologies for breeding and plant protection, as well as provide a broad perspective on the risks, challenges, public perception, and regulatory aspects concerning the applications of non-transgenic approaches of both genetic engineering technologies in modern agriculture. In **Figure 1**, we summarized the main risks and challenges related to both technologies, which will be discussed further in this review.

2 GENOME EDITING TECHNOLOGY FOCUSES ON CRISPR/CAS TECHNOLOGY

2.1 CRISPR/Cas in Agriculture

CRISPR/Cas has been used in different crops since 2013, introducing into them agricultural traits of great value, such as

yield, quality, and biotic-/abiotic-stress resistance (Shan et al., 2013; Zhang et al., 2020). This technology holds an enormous potential to address numerous concerns involving cost, time, and complex biosafety issues, typical characteristics of the transgenic strategy. Furthermore, the ever-expanding CRISPR/Cas toolbox has allowed a myriad of applications in plants, including the knockout and knock-in of target genes, modulation (i.e., inhibition or activation) of gene expression, genome base editing, among others (Zhu et al., 2020).

Differently from other genome editing-based technologies, such as zinc-finger nuclease (ZFN) and transcription activator-like effector nuclease (TALEN), the use of CRISPR/Cas does not depend on engineered proteins, and it is essentially based on RNA/DNA hybrids, in which its target specificity relies on a short stretch of RNA, providing higher versatility, lower costs and an easier building process. Furthermore, this technology enables the editing of multiple genome sites simultaneously (Xiong et al., 2015), and also introduces mutations directly into elite crop varieties, bypassing limitations like narrowed natural genetic variability, and time-consuming processes of backcrossing to reconstruct the elite genetic background as in conventional breeding technique (Scheben et al., 2017; Rato et al., 2021), being especially useful for crops with rare resistance sources, long life cycles, and polyploid genomes.

The gene regulation can be modulated by the use of catalytically inactive Cas9 variants (e.g., dead Cas9—dCas9) or orthologs. These enzymes are capable of binding to specific DNA sequences mediated by gRNA without causing double-strand breaks on the DNA molecule. (Lowder et al., 2018; Papikian et al., 2019). The dCas9 fused to transcription regulatory domains, such as VP64 or SRDX, or epigenetic modulators can be used for activation or repression through CRISPR interference (CRISPRa or CRISPRi, respectively), expanding its range of applications (Moradpour and Abdulah, 2020). For example, dCas9-VP64 and dCas9-TV systems increased the expression of the UDP-glucose flavonoid glycosyl-transferases (UFGT) gene in grape cells (Ren et al., 2022). dCas9 can also promote/inhibit enhancers in promoter regions of genes due to the interference in chromatin structure, consequently modulating the gene expression (Morgan et al., 2017). Another way of modifying the gene expression is the cleavage and degradation of RNA-targeting using Cas13a and Cas13b. Editing RNA with CRISPR/Cas13 is a novel and emergent tool in plants and is currently being used mainly for developing resistance to viral diseases in plants (Zetsche et al., 2015).

Beyond the coding (CDS) and promoter regions, other regulatory elements are also good targets for genome editing aiming to modulate gene expression, such as polyadenylation signals, alternative transcription initiation sites, and upstream open reading frames (uORFs). Usually responsible for reducing the translation, uORFs are situated in the 5′ untranslated regions (UTRs) of mRNAs, and when edited can promote the upregulation of gene expression. For example, the CRISPR knockout of gene's uORF region resulted in an increase in gene translation in lettuce (*Lactuca sativa*) and strawberry (*Fragaria vesca*) plant crops, leading to a high content of ascorbate and sweetness in the edited plants, respectively (Zhang et al., 2018; Xing et al., 2020).

2.2 Risks and Challenges Involving CRISPR/Cas Technology

2.2.1 Unintended Off-Target Effects (General Immune Response)

During the activity of CRISPR/Cas machinery, the gRNA can direct the Cas protein to other regions and consequently lead to unintentional cleavage of DNA sequence, a process known as off-target effect. Shahriar et al. (Shahriar et al., 2021) classified the off-targets into two types: 1) sequences sharing high similarities to the target, and 2) irrelevant genomic off-target sites. These off-target mutations are of great concern mainly in the clinical-therapeutic area (Zhang et al., 2015), which restricts its application due to technical and ethical issues. In major crop plants, several studies have reported the incidence of unwanted changes in the genome, but at low rates (<10%) (Peng et al., 2017; Tang et al., 2018; Young et al., 2019; Graham et al., 2020; Jin et al., 2021), suggesting a remarkable specificity of CRISPR/Cas system in the plant genome, or either a flaw in the currently available off-target detection methods (Bortesi and Fischer, 2015; Hajiahmadi et al., 2019). Nevertheless, when an off-target effect is detected, it is generally located at genomic spots exhibiting great similarity to the target sites (Lawrenson et al., 2015; Tang et al., 2018). Some *in vitro* and *in vivo* methods have been developed to detect these mutations, such as Digenome-seq (Kim D. et al., 2015), GUIDE-seq (Tsai et al., 2014), SITE-seq (Cameron et al., 2017), CIRCLE-seq (Tsai et al., 2017), and DISCOVER-seq (Wienert et al., 2019). A gold standard recommendation would be performing genome-wide NGS for the identification of these potential off-target mutations, however, it seems not to be practical/feasible in most cases (Hahn and Nekrasov, 2019; Shillito et al., 2021), especially for polyploid crops. Consequently, an underestimation of off-target mutation rates might be occurring, although not likely.

2.2.2 Epigenetic Consequences

Epigenetic phenomena consist of a complex gene expression regulation process for the maintenance of a precise state of gene activation/repression in a given cell (Urnov and Wolffe, 2001). Such a sophisticated and fine-tuned mechanism involves a series of alterations in DNA molecules, including chemical modification of DNA structure (e.g., methylation), modification in histone proteins (closely associated with the gene locus), and chromatin remodeling, without altering DNA primary sequence (Jaenisch and Bird, 2003). Although epigenetic characteristics can influence cleavage by facilitating or hindering DNA accessibility, unintended effects on the genome beyond off-target mutations caused by the use of CRISPR technology are still poorly explored. Lee et al. (2020) analyzed the DNA methylation profiles in promoters of naturally hyper and hypomethylated genes from *Arabidopsis thaliana* that underwent genome editing through CRISPR/Cas. Edited and wild-type plants showed the same epigenetic profile by sequencing the next generation of bisulfite-converted DNA, concluding that CRISPR genome editing did not result in unintended epigenetic changes. However, only one work was carried out in the area and one epigenetic mechanism was evaluated. DNA methylation is the

most common epigenetic marker in plants and occurs mainly by the insertion of a methyl group (CH_3) on the fifth carbon of cytosines in CpG (cytosine-phosphate-guanine) dinucleotides (Laird, 2010; Yong et al., 2016). Meantime, epigenetic information is also mediated by post-translational histone modifications (MPTHs) and processing mechanisms of non-coding RNAs (ncRNAs) (Bossdorf et al., 2010).

Another important point to be raised is the DNA accessibility in target regions by CRISPR technology. Studies have shown that the level of accessibility to the loci through DNA methylation or chromatin structure can influence the efficiency of on-target gene editing (Jensen et al., 2017; Verkuijl and Rots, 2019; Strohkendl et al., 2021). The chromosome with high compaction can lead to low DNA accessibility to non-specific Cas9 interactions (Chitra et al., 2019). Nucleosomes are known to inhibit PAM (protospacer adjacent motif) site recognition, reducing the rates of Cas nuclease cleavage *in vitro* (Verkuijl and Rots, 2019; Strohkendl et al., 2021). Additionally, there is a positive correlation between chromatin opening and the efficiency of mutagenesis by the CRISPR system (Uusi-Mäkelä et al., 2018). For example, a transcriptional activation domain fused to Cas9 improved the genome editing efficiency in condensed and relaxed chromatin regions in rice (Liu et al., 2019). Given the above, in addition to PAM recognition and complementarity between gRNA and target DNA, DNA accessibility should also be considered an important factor for genome editing efficiency.

2.2.3 Toxicity Impacts on Human/Animal Health

The toxicity associated with CRISPR/Cas application may be caused by its components, the exposure period, and/or depending on the delivery methods. Several studies involving different organisms, such as prokaryotes (Jiang et al., 2014, 2017; Cho et al., 2018; Markus et al., 2019) and pluricellular eukaryotes (Ihry et al., 2018; Li et al., 2018; Rosenblum et al., 2020), have shown that either induced double-strand break (DSB) and heterologous Cas9 protein expression can impair cell growth that leads to an abnormality in cell morphology and/or trigger cell death. To date, no reports of Cas9-associated toxicity have been found in plants (Dey, 2021). Since the first applications of CRISPR technology in plant cells, researchers have shown that whole plants can be regenerated by tissue culture from edited cells, suggesting that the CRISPR system components are not toxic to plants (Hahn and Nekrasov, 2019). However, depending on the adopted CRISPR/Cas strategy and the target chosen, serious pleiotropic effects may occur (Zhu et al., 2020). For example, the knockout of plant susceptibility (-*S*) genes, associated with pathogen compatibility, but also engaged with multiple crucial pathways, often lead to plant fitness penalties, including physiological and growth tradeoffs (Kale et al., 2019; van Butselaar and Van den Ackerveken, 2020; Kieu et al., 2021). In addition, studies have evaluated the toxicity of CRISPR/Cas components not only in plants but also in humans. Regarding the exposure of Cas protein in humans, an interesting study conducted by El-Mounadi et al. (El-Mounadi et al., 2020) concluded that exposure of human beings to Cas9 proteins took place long before the emergence of genomic editing tools. In the comparative genomic analyses, the authors detected more than 80% similarity between *Streptococcus pyogenes* (*Sp*Cas9) amino acid sequence with commensal/pathogenic bacteria such as *Streptococcus dysgalactiae* subsp. *equisimilis*, *Staphylococcus aureus*, *Klebsiella pneumonia*, and *Streptococcus canis*, are commonly found in the environment or even in foods intended for human consumption. Furthermore, *Sp*Cas9 has homologs in Gram-positive and Gram-negative bacteria naturally found in different niches throughout the human body (Louwen et al., 2014). Hence, edited plants containing Cas9 integrated into the genome probably do not represent a potential risk to human health.

Nevertheless, the mode of delivery of CRISPR/Cas system components seems to stand out as one of the main factors of toxicity in plants. For example, nanoparticle (NP)-based delivery approaches for the transfection of CRISPR reagents, while representing a promising association as will be addressed later in this review, toxicity concerns have been raised (Demirer et al., 2021). As such, systemic toxicity studies have suggested that the physical and chemical properties of nanomaterials must be taken into account. For example, in the case of the carbon nanotube, in which limitations of its use have been emphasized due to the non-biodegradable nature and the presence of heavy-metal impurities introduced during NPs synthesis (Kostarelos, 2008; Pikula et al., 2020). In this setting, to avoid future problems in the United States, the application of new substances as nanocarriers in agriculture must demonstrate safety and absence of toxicity effects before its application in the field, following the regulation of the Toxic Substances Control Act (TSCA) law (Heller et al., 2020). Furthermore, the generation of data about nanomaterial's lifecycle in CRISPR/Cas edited plants and their progeny, its fate in the environment, likewise the potential impacts on interacting organisms, including humans, may provide crucial information towards the approval of new, safer, and more sustainable NPs (Demirer et al., 2021).

2.3 Strategies to Increase On-Target Specificity/Efficiency and Avoid Toxicity in Plants

Efforts have been made toward the optimization of CRISPR/Cas strategies to increase on-target specificity/efficiency as well as reduce off-target effects and toxicity in plants (Hajiahmadi et al., 2019). In general, the main technical factors that may influence undesirable outcomes in plants are the gRNA design, choice of Cas variant proteins, specific CRISPR component formats, and the delivery methods of CRISPR/Cas reagents into the target genome.

2.3.1 Properties and gRNA Design

A prerequisite for reducing off-target effects is optimizing the gRNA design, and carefully selecting the sequence to be targeted (Hsu et al., 2013; Zhu et al., 2017; Zischewski et al., 2017). Bioinformatics web-based tools have been developed for the gRNAs design and to predict potential off-target sites in plant genomes, including Cas- OFFinder (Bae et al., 2014), CHOPCHOP v.2 (Labun et al., 2016), CRISPOR (Haeussler et al., 2016), CRISPR-P 2.0 (Liu et al., 2017), CRISPR-GE (Xie et al., 2017), CRISPR-PLANT v.2 (Minkenberg et al., 2019), and

CRISPR-BETS (Wu et al., 2022). For more details, refer to Gerashchenkova et al. (Gerashchenkova et al., 2020), which describes over a hundred software for gRNAs design. Hahn and Nekrasov (Hahn and Nekrasov, 2019) emphasize that the species having annotated genome sequence available is not mandatory, but necessary for an effective prediction once it would allow examining off-targets also located in non-coding regions. In summary, these above-mentioned tools consider incompatibilities within the gRNA seed sequence (8–12 nucleotides upstream to PAM), being its number and position decisive for gene editing specificity. In addition, mismatches located between the eight nucleotides proximal to the PAM site reduce off-target effects. According to Modrzejewsk and co-workers, the off-target effect rate decreases 59% if there is a unique mismatch between the target and off-target sequence. In the cases that there are four or more mismatches, this value reduces further to 0.09% (Modrzejewski et al., 2020).

Another factor to consider for enhancing gRNA specificity is the ratio of guanine-cytosine (GC) nucleobases, even though there is no consensus among the studies. The hypothesis is that a low GC content decreases off-target occurrence (Yu et al., 2017), as the high content stabilizes the hybridization of gRNA to genomic DNA (Fu et al., 2013). While some studies have shown that gRNA sequences with low (<20%) or high CG (>80%) content are less effective against targets (Wang et al., 2014b; Ma et al., 2015), others did not identify interference from the total GC content of gRNA (Ren et al., 2014; Jensen et al., 2017; Labuhn et al., 2018; Modrzejewski et al., 2020). Recently, the study by Malik et al. (Malik et al., 2021) showed that the high GC content in the seed region (1–12 nucleotides close to PAM) decreases the activity of gRNAs, negatively influencing the target cleavage efficiency. So, the use of intermediate GC contents (~50%) is indicated as a reference for gRNA design to improve the on-target specificity. However, more studies are needed to better elucidate how it operates.

2.3.2 Cas Protein Variants

Limitations for CRISPR technology using *Sp*Cas9 include protein size, off-target effects, and the requirement of a specific PAM sequence (NGG) in the genome, which restrain potential target recognition sites (Zhi et al., 2021). Two main approaches have been adopted as alternatives to overcome this restriction: the use of Cas9 orthologs derived from different organisms and the Cas9 protein modification to recognize different PAM sequences (Sukegawa et al., 2021). For a full list describing natural and engineered Cas nuclease variants used in genomic editing, refer to Anzalone et al. (Anzalone et al., 2020).

Natural Cas9 variants presenting different PAM sequences, such as those from *Staphylococcus aureus* (*Sa*Cas9—NNGRRT), *S. thermophilus* (*St1*Cas9—NNAGAAW, W = A/T), and *S. canis* (*Sc*Cas9—NNG) had their specific recognition sites demonstrated in different plant experiments (Steinert et al., 2015; Kaya et al., 2016; Wang M. et al., 2020; Veillet et al., 2020). Numerous engineered variants have also been developed (*Sp*Cas9-VQR, *Sp*Cas9-EQR, *Sp*Cas9-VRER, *Sp*Cas9-NG, *Sp*Cas9-HF1, *eSp*Cas9, *Hypa*Cas9, *evo*Cas9, *Sniper*-Cas9, *x*Cas9, and *Sp*RY) based on the crystal structure of Cas9 attached to gRNA and target DNA (Wada et al., 2020). These natural and engineered variants exhibit relaxed PAM sites, smaller sizes compared to *Sp*Cas9 (1,368 aa), high target specificity, and promising applications (Negishi et al., 2019; Qin et al., 2019; Xu et al., 2019; Zhong et al., 2019).

The nuclease Cas12a (previously called Cpf1), widely used for genome editing in plants, opened the possibility to target adenine-thymine-rich genomic regions (Zetsche et al., 2015). Cas12a has a PAM sequence rich in "T" nucleotides (TTTV, V = A/G/C). Unlike Cas9, Cas12a has two RuvC catalytic sites, its cut generates blunt ends in the DNA double-strand, and it does not have tracrRNA (trans-activating CRISPR RNA) in the system. These properties make this nuclease more suitable for generating larger deletions and multiplex gene editing (Zhang et al., 2021b; Huang Holger Puchta et al., 2021).

The dCas9 engineered variant enzyme is able to alter the phenotype (e.g., modulating gene expression and/or translation) without changing the genetic code of plants, thus representing an interesting alternative approach to reduce off-target effects, bypass DSB-induced toxicity, avoiding pleiotropic and lethal effects in the targeted plant (Lei et al., 2013; Brezgin et al., 2019). The use of two dCas9 simultaneously at the same locus to cleave each DNA strand has also been proposed to reduce potential off-target effects (Pereira, 2016).

2.3.3 Alternative CRISPR Component Formats

In general, plasmid DNA expression vectors harboring a CRISPR gene cassette are used in the genetic transformation of target organisms via *Agrobacterium tumefaciens* (Das et al., 2021) or through particle bombardment (Imai et al., 2020). However, this most frequently applied strategy has as major concerns the random integration into the genome and the continuous expression of Cas protein and gRNA(s), which increases the possibility of chimeric mutants, off-target effects, and toxicity (Feng et al., 2014; Hashimoto et al., 2016). To overcome these issues, the availability of different CRISPR/Cas system reagent formats, such as mRNA and pre-assembled RNPs, represent promising alternatives (Liang et al., 2017). RNP-based DNA-free genome editing in plant cells usually occurs through PEG, electroporation, lipofection, and particle bombardment (Zhang et al., 2021a). After delivering the complex into the cell nucleus, RNP is rapidly degraded, thus avoiding potential off-target effects (Kim J.-S. et al., 2015, 2017; Subburaj et al., 2016). Moreover, for cellular toxicity associated with long-term expression of Cas and/or integration of exogenous DNA, the RNP complex approach may represent a good choice due to the transient and stable transfection in the plant cell (González et al., 2021). On the other hand, as this strategy does not use selection marker genes, the screening of edited plants with desirable phenotypes may become more laborious and costly. Additionally, this method often presents lower editing efficiencies (~10%) compared to stable integration vectors, as already demonstrated for corn (*Zea mays*) (≤9.7%) (Svitashev et al., 2015), brassica plant species (≤24.51%) (Murovec et al., 2018), potato (*Solanum tuberosum*) (≤25%) (Andersson et al., 2018), and petunia (*Petunia juss*) (≤11.9%) (Yu et al., 2021).

2.3.4 The Use of Viral Vectors and the Association With Nanomaterials

The success of CRISPR technology relies directly on the approach used to deliver its reagents. However, the cargo of biomolecules consists of one of the main steps and bottlenecks in genetic transformation. Unlike animals, plant cells possess a cell wall that represents a natural physical barrier limiting the entrance of exogenous molecules into the cytoplasm. Biolistics and *A. tumefaciens* transformation are the conventional methods typically used to overcome plant cell wall, but these approaches have several disadvantages that can negatively impact the transformation process, such as low efficiency of target edition, plant tissue damages, and technical incompatibilities (Altpeter et al., 2016; Demirer et al., 2021). Notwithstanding, the recent advancements in the field of delivery using viral vectors and nanoparticles have delineated new possibilities, surpassing traditional limitations and contributing to improvements in the genetic engineering of plants (Cunningham et al., 2018).

Some viruses are efficient in the genomic editing of plants due to their ability to infect and replicate into the cells of a wide range of plant species (Zhu et al., 2020). Over the years, there have been remarkable advances in virus research as carrier agents involved in plant genome editing, also known as virus-induced genome editing (VIGE) (Gentzel et al., 2022). In principle, only engineered RNA or single-stranded DNA viruses positive-sense were used to express gRNA strands, however requiring the expression of Cas protein in genetically modified plants for the effectiveness of the CRISPR/Cas system (Ali et al., 2015a, 2015b; Yin et al., 2015; Oh et al., 2021). Later, it is possible to stably express Cas and gRNA in single-stranded RNA virus negative-sense. For example, *Barley yellow striate mosaic virus* (BYSMV) and *Sonchus yellow net virus* (SYNV) were able to efficiently edit *Nicotiana benthamiana*, but without transgenerational effect due to the inability of these viruses to penetrate the meristematic and reproductive tissues of the plant (Gao et al., 2019; Ma and Li, 2020). Currently, the fusion of mobile elements to the gRNA in the infection clone has belonged to the presence of the virus in meristematic tissue, consequently inducing the mutation in the progenies (Ellison et al., 2021; Lei et al., 2021).

In theory, all available formats of CRISPR/Cas system reagents (e.g., plasmid DNA expression vectors, mRNA, and RNP complexes) can be encapsulated in nanomaterials prior to cell delivery. Nanomaterials can improve cellular uptake, as well as circumvent technical limitations, such as the low stability of CRISPR reagents depending on the format chosen (Demirer et al., 2021). A multitude of NPs have been developed and tested in an attempt to improve the transformation efficiency of different crops, however few of them have been successful as carriers of CRISPR/Cas system components. The most common nanomaterial tested to deliver DNA and other chemical reagents in plant cells is mesoporous silica (Torney et al., 2007). Other well-known NPs are carbon nanotubes, which are passively absorbed by plant cells without being degraded by endonucleases (He and Zhao, 2019). Promising results using these NPs to nanoencapsulation and deliver plasmid DNA into chloroplast organelles have been reported in brassica, cotton (*Gossypium hirsutum*), and wheat (*Triticum aestivum*) (Kwak et al., 2019; Demirer et al., 2020). Likewise, layered double hydroxides (LDHs) and carbon dots are also a good choice of NPs, once they can penetrate plant cells causing minor injuries and efficiently protecting the internalized content (Bao et al., 2017). Doyle et al. (Doyle et al., 2019) performed one of the few studies in the literature reporting the use of NP to deliver CRISPR/Cas components to plant cells. Authors showed that naturally occurring carbon dots (quasi-spherical, <10 nm nanoparticles) can be used as a vehicle for carrying Cas9 and gRNA plasmid coated carbon dots into wheat plants via foliar application by spraying and to generate target mutations. Instead, Sandhya et al. (Sandhya et al., 2020) suggest the direct delivery of RNPs to regenerative tissues using a pollen magnetofection-mediated delivery. The methodology aims to use pollen as a nanocarrier agent for exogenous DNA molecules, and later the use of this pollen to fertilize the plant's ovary and directly induce the genetic edition of seeds.

Altogether, the rapid evolution of CRISPR/Cas technology and all associated-approaches/strategies available for plant genome editing provide optimal conditions to target the above-mentioned technical-related challenges and also to improve the understanding of risk/safety implications. Lastly, although concerns about unintended off-target effects and potential toxicity have raised discussions around CRISPR adoption in plant breeding, these should not be considered criteria for restricting CRISPR technology application, as in the case of its usage in animal cells, apparently. Moreover, in most plant species it is possible to eliminate off-target mutations and inferior traits through genetic segregation by the backcross breeding approach (Murovec et al., 2018).

3 RNAI PLANT-BASED TECHNOLOGIES

RNAi-based transgenic plants, designed to express dsRNA sequences to knock down the expression of specific genes in the host and/or pathogen genome, have represented a remarkable complementary tool to face the abusive usage of pesticides in agricultural fields, with great potential to cause environmental and human health problems (Mezzetti et al., 2020). However, in the last few years, the global demand for a more sustainable and non-transformative technologies of crop protection has substantially intensified (Budzinski and Couderchet, 2018; Fletcher et al., 2020). In this context, the scientific community has strived to develop and master the application of novel non-transgenic RNAi-based technologies.

3.1 Topical RNAi-Based Approach Towards a More Sustainable Plant Protection

The breakthrough and Nobel Prize-winning discovery that oral delivery of dsRNA to *C. elegans* induced a potent and specific gene silencing (Fire et al., 1998), nourished the perception that exogenous dsRNA application could trigger RNAi response on

any target organism, and paved the way for the emergence of the topical RNAi-based technology. Such approach consists of producing high amounts of self-delivering dsRNAs to be topically used in the field as bio-defensive molecules (Das and Sherif, 2020), so far standing as a promising tool in agriculture to achieve plant protection against several pathogens (Dalakouras et al., 2020; Kiselev et al., 2022).

Tenllado and Diaz-Ruiz (Tenllado et al., 2003) and Tenllado et al. (Tenllado et al., 2003) were the first to report the plant protection from viruses by topical dsRNA application. They showed the foliar application of *in vitro* expressed dsRNA molecules targeting the plant viruses *Pepper mild mottle virus* (PMMoV), *Plum pox virus* (PPV), *Alfalfa mosaic virus* (AMV), and *Tobacco etch virus* (TEV) conferred plant resistance against infections. Following this pioneering discovery, different studies reported successful control of multiple families of plant viruses by topical RNAi-based technology (Mitter et al., 2017).

Similar to viruses, fungal control by topical application of dsRNAs seems to be promising. Koch et al. (Koch et al., 2013) showed that *in vitro* cultures of *Fusarium graminearum* treated with dsRNAs complementary to three cytochromes P450 (CYP) genes resulted in growth inhibition, similarly to the observed after treatment with fungicide tebuconazole. Also, they reported that topical application of these dsRNAs on detached barley leaves impaired *F. graminearum* growth beyond the applied sites, suggesting a systemic activity (Koch et al., 2016). Moreover, the surface of fruits, vegetables, and flowers sprayed with dsRNAs targeting two DICER-LIKE (DCL) genes of *Botrytis cinerea* resulted in effective control of the pathogen, demonstrating that topical RNAi-based approaches may be useful to protect crops either during the production cycle as in post-harvest stages (Wang et al., 2016).

The first demonstration of exogenous dsRNA application against insect pests came from a study on citrus and grapevines to control two hemipteran pests, the xylem-feeding glassy-winged sharpshooter *Homalodisca vitripennis*, and the phloem-feeding psyllid *Diaphorina citri*. Both insects tested positive for dsRNA ingestion after feeding on plants treated with dsRNAs applied to the root zone, showing the movement of dsRNA through the graft junction of rootstock and scion (Hunter et al., 2012). Later, San Miguel and Scott (San Miguel and Scott, 2016) demonstrated the dsRNA application on leaves of potato plants targeting the actin gene on Colorado potato beetle (*Leptinotarsa decemlineata*) resulted in significant mortality of insects. Moreover, the result showed that dsRNA remained biologically active on potato leaves for at least 4 weeks under greenhouse conditions.

Although mounting evidence demonstrates the efficacy of topical RNAi-based technology to enhance quantitative and qualitative valuable agronomic crop traits, relevant concerns have been raised about its feasibility, from delivering methods to relative costs of the technology, as well as the associated risks. All these matters must be overwhelmed to allow a straightforward translation of research data into new biotechnological commercial solutions, intending to minimize environmental, health, and regulatory issues.

3.2 Risks and Challenges Involving Topical Application of dsRNA

Topical RNAi-based technologies offer clear benefits over most existing crop protection chemical pesticides. However, an approach based on scientific parameters to develop and validate procedures is fundamental to defining which risk assessment criteria are most appropriate for these technologies (Mezzetti et al., 2020).

3.2.1 Weighting the Unintended Off-Target Effects

Usually, off-target effects are due to the existence of any degree of sequence similarity between siRNA (e.g., synthetic and/or derived from dsRNA processing by DICER enzyme) and non-target mRNA transcripts (Chen et al., 2021a). In this context, the presence and position of nucleotide mismatches along with the siRNA molecule structure, in relation to the target sequence, seem to exert a major influence in the silencing of nontarget genes. Kulkarni et al. (Kulkarni et al., 2006) investigated dsRNA specificity using the model insect *Drosophila melanogaster*. Through a high-throughput screening, authors reported that long dsRNAs sharing a perfect identity of as few as 19 nt-long with predicted unintended targets, lead to off-target effects. Investigating governing rules of dsRNA specificity in the beetle *Tribolium castaneum*, Chen et al. (Chen et al., 2021b) showed that a dsRNA targeting a member of the CYP, the gene CYP6BQ6, was able to silence another eight genomic regions with nucleotide sequence identity ≥68%. Among these genes, CYP6BK7 and CYP6BK13 showed significant alteration in transcript modulation. Sequence analysis found that CYP6BK7 and CYP6BK13 contain 24 and 26 bp of contiguous matching bases with only two single mismatched bases, respectively. Further investigations using mutational analyses showed that dsRNAs with ≥16 bp perfectly matched sequence or >26 bp almost perfectly matched sequence (i.e., with one or two mismatches scarcely distributed) were also able to trigger RNAi gene silencing on *T. castaneum* off-target transcripts. Taning et al. (Taning et al., 2021a) used a sequence complementarity-based approach to evaluate potential off-target effects in bumblebee (genus *Bombus*), following oral exposure to a chimeric dsRNA. Interestingly, no modulation was found in the transcript level for all potential predicted off-targets, including sequences with 20 continuous nucleotide matches or with 21 bp stretch with only one mismatch.

Besides the important role of nucleotide mismatches, as well as the apparent variation in the occurrence of off-target gene silencing between organisms, two other ways may trigger off-target activity. First, the RNAi enzymatic complex (more specifically the Argonaute RISC Catalytic Component 2-AGO2 enzyme) can erroneously incorporate the wrong strand of siRNA sequence (e.g., the passenger strand) leading to the downstream degradation of unintended transcripts (Schwarz et al., 2003). The second and unpredictable triggering of off-target activities may occur if the small RNA binds to the miRNA pathway, which can result in the silencing of dozens if not hundreds of transcripts (Doench et al., 2003; Jackson et al., 2003).

3.2.2 Cross-Kingdom Nontarget Risks and Related Biosafety Issues

Considering that off-target effects are usually surveyed only within target organisms, very little is known about how dsRNAs affect the gene silencing in nontarget organisms. Suffice to say that, aiming at the generation of RNAi-based technological solutions for agriculture, the risk analysis should encompass each of the myriad interacting organisms in the agroecosystem, including humans, that may be directly or indirectly exposed to the dsRNA molecules.

The fact is that cross-species and cross-kingdom nontarget effects may occur more often than is commonly argued. For example, Zhang et al. (Zhang et al., 2011) reported a quite intriguing result showing that a great amount (up to 10%) of plant exogenous microRNAs were found in sera and tissue samples of various animals and that these are likely taken orally with food. MIR168a is a plant miRNA very abundant in rice crops. Surprisingly, the amount of MIR168a was found to increase in the serum of rats fed with a rice-containing diet, even when it was cooked. Following *in vitro* and *in vivo* functional assays showed the ability of rice MIR168a to bind both to human and mouse transcripts, resulting in non-target gene silencing effects. Another interesting study demonstrated that dsRNAs expressed by transgenic maize crops, and designed to silence target genes in the western corn rootworm (Baum et al., 2007). *Diabrotica virgifera* also impacted the expression of orthologous gene members present in the other insect species, *D. undecimpunctata* and *L. decemlineata*, despite the relatively low sequence homology between genes in the target and nontarget organisms.

In terms of cross-kingdom dsRNA transference, recent studies have shown the transference of small RNAs between plants and pathogens occurs spontaneously in nature, participating mainly in defense mechanisms (Guo et al., 2018). For example, cotton plants produce miRNAs (e.g., miR166 and miR159) which are exported directly to the hyphae of the fungus *Verticillium dahliae*, a vascular pathogen responsible for wilt in many cotton crops, targeting transcripts engaged with fungus's virulence, and conferring plant resistance (Zhang et al., 2016). Likewise, it was described that small RNAs (e.g., TAS1c-siR483 and TAS2-siR453) produced by the model plant *A. thaliana* were detected in cells of the fungus *B. cinerea* during plant-pathogen interaction, and plant lines overexpressing these small RNAs displayed reduced susceptibility to this pathogen, which showed a negative modulation of targeted transcripts (Cai et al., 2018).

Similarly, small RNAs can also be transmitted in the opposite direction, i.e., from the pathogen to the host plant. One of the first studies that demonstrated the transfer of small RNAs from pathogens to plants was performed by Weiberg et al. (Weiberg et al., 2013), where three siRNAs from the fungus *B. cinerea* (Bc-siR3.1, Bc-siR3.2, and Bc-siR5), with predicted targets in *A. thaliana* and tomato (*Solanum lycopersicum*) plants, rendered both host plants susceptible to fungus infection. Furthermore, *Arabidopsis AGO1* mutants, unable to process the small RNAs from *B. cinerea*, exhibited reduced susceptibility to the fungus, as well as to *B. cinerea* DCL1/DCL2 double mutant, which exhibited reduced pathogenicity on both plants.

The natural traffic and delivery of these small RNA molecules inside and between interacting plant-pathogen organisms can be done through extracellular vesicles (EVs), i.e., membrane-bound particles that carry manly transmembrane proteins and RNAs, being produced by both sides of the pathosystem (Liu et al., 2021). In plants, stress-associated EVs were isolated and characterized in apoplast fluids from *Arabidopsis* leaves, from where they are assimilated by the pathogen/pest (Rutter and Innes, 2017). Different studies on the *A. thaliana* and *B. cinerea* interaction have demonstrated the transfer from plant to fungus of "tiny RNAs," which are 10–17 nucleotides in length, and derived mainly from the positive strand of mRNA transcripts (Cai et al., 2018; Baldrich et al., 2019). On the other direction, recent studies have also reported the EVs delivery from pathogens to plants. Bleackley et al. (Bleackley et al., 2020) demonstrated that EVs secreted by the fungus *F. oxysporum* induced phytotoxic responses in cotton plants. Likewise for the fungus *Zimoseptoria tritici*, whose EVs are engaged with the triggering of pathogenesis in wheat crops (Hill and Solomon, 2020).

Based on the studies of cross-kingdom small RNA transfer, small RNAs exchanged between plants and pathogens could have five possible fates: 1) if the expression is not sufficient and the concentration of small RNAs is low, the transferred dsRNA could be diluted during proliferation and division of the recipient cell; 2) RNAi-mediated signaling can be amplified by the production of secondary siRNAs; 3) the transferred RNAs can be degraded by RNAi suppressor proteins; 4) long dsRNAs can activate RNAi system and induce gene silencing in recipient plant and; 5) the transferred RNAs can improve the adaptability of recipient plants to the environment and it can be retained and fixed in the genome of the recipient plant through horizontal gene transfer (Zhao et al., 2021).

3.2.3 Challenges Related to the Uptake and Stability of Topically-Applied dsRNA

The advantages of topically-applied dsRNA and its potential as a biopesticide commercial product are still hindered by technical issues, including molecule uptake and stability, delivery methods, inconsistent activity of the dsRNA trigger, and activity level of RNAi suppression (Hunter et al., 2021). Hence, one of the first aspects that should be addressed when thinking about topical RNAi-based technology is the uptake efficiency of dsRNAs and/or siRNAs/miRNAs either by the pathogen or plants, depending on the adopted strategy. In the case of having phytonematodes as a target for gene silencing, self-delivering dsRNA molecules are ideally supplied as food nearby the plant root *in vivo* assay and the uptake is made by pathogen's ingestion. Once in the midgut cells of the nematode, the molecule internalization is mediated by several transmembrane proteins, known as systemic RNA interference deficiency (SID), in particular, the proteins SID-1 and SID-2, triggering a systemic gene silencing (Winston et al., 2002; Wang and Hunter, 2017; Whangbo et al., 2017). For insects, there are two described mechanisms of dsRNA uptake: 1) likewise mediated by SID-like proteins (SIL), and 2) clathrin-mediated

classical endocytosis. Different studies on the dsRNA uptake by *Apis mellifera* (Aronstein et al., 2015), *D. virgifera* (Miyata et al., 2014), and *L. decemlineata* (Cappelle et al., 2016) reported that either the overexpression or knockdown of SIL genes caused variations in gene silencing, while in *Plutella xylostella* (Wang et al., 2014a), *Schistocerca gregaria* (Wynant et al., 2014) and *Tribolium castaneum* (Tomoyasu et al., 2008), the knockdown of those genes did not affect uptake efficiency (Xu and Han, 2008; Bansal and Michel, 2013). Moreover, the molecular process generally involves the recognition of dsRNAs by scavenger receptors, which can be influenced by the length of dsRNA molecules, as demonstrated for species of the order Coleoptera, where very small dsRNAs were not effectively internalized (Miller et al., 2012). Although these represent the most accepted and well-described models for dsRNA uptake in insects, it is still not known why dsRNA remains within the endosomes of some species of the order Lepidoptera, which directly influences the efficiency of RNAi-mediated gene silencing (Yoon et al., 2017). For other disease-causing agents of plants, such as phytopathogenic fungi, the knowledge about dsRNA uptake mechanisms is still limited. However, a recent study with *Sclerotinia sclerotiorum* suggested that dsRNA uptake is mediated via classical clathrin endocytosis, likewise for insects, but dsRNA recognition receptors remain elusive (Wytinck et al., 2020).

Ultimately, it is reasonable that dsRNA uptake efficacy may vary due to numerous factors, including differences in insect feeding behavior, its availability on feeding sites, lack of gene silencing amplification signal, and also dsRNA degradation during ingestion (Niu et al., 2019). Furthermore, even though *in vitro* assays involving the oral feeding of pathogen and/or disease-carrying insect vectors with dsRNAs targeting their essential genes have been shown to induce consistently high mortality, reproducing these results on the field conditions by topical application strategy represents a great challenge.

Another approach to improve plant crop resistance consists in the foliar application of self-delivering dsRNAs to the plant surface, aiming at the knockdown of plant genes whose expression is associated with pathogen susceptibility. Such strategy is likewise crucial to ensure that topically-applied dsRNA display both appropriate stability, to hinder dsRNA premature degradation by environmental factors (e.g., rainwater, sunlight/UV radiation, and microorganisms), and a great capacity to penetrate the natural plant foliar barriers, such as waxy cuticles, trichomes, and the cell wall (Bennett et al., 2020; Rank and Koch, 2021). Therefore, due to these significant challenges, there are still very few studies reporting the success of this approach. Dubrovina and co-workers (Dubrovina and Kiselev, 2019) showed that a prior foliar cuticle abrasion through a high pressure using microparticles may facilitate dsRNA absorption by plant cells. Likewise, the use of surfactant agents has been shown to improve dsRNA entrance through the foliar stomatal aperture (Bennett et al., 2020).

Furthermore, studies have shown that dsRNA molecules are degraded very rapidly in the environment (Bachman et al., 2020). Therefore, another point to be addressed is the increase in dsRNA protection window, which is very short when applied "naked," limited to a few days in the environment (Rego-Machado et al., 2020). In the case of topical RNAi-based products, in which dsRNAs may be conjugated with nanoformulations to increase their absorption, and stability, among other parameters, a case-by-case risk assessment should be required (Mendelsohn et al., 2020).

3.3 Strategies to Increase On-Target Specificity, Stability, and Delivery of Exogenous dsRNA

3.3.1 dsRNA Molecule Design

The accumulated experimental data is helping to increase the accuracy of prediction models and RNAi design tools, which allows inferences about the efficiency of the dsRNA *in silico*. To obtain the greatest efficiency of the RNAi technology, three factors must be taken into account: 1) the number of siRNA generated from a single dsRNA; 2) the specificity of the siRNA to the target transcript, and 3) chemical alteration in the seed region of the siRNA guide strand. The enzyme DICER endonuclease attaches to longer dsRNAs, resulting in the accurate cleavage of dsRNAs into shorter siRNAs. The presence of the DICER cleavage site increased effectiveness up to 100-fold compared to a sequence without the site (Cooper et al., 2021). It has also been proposed that apart from the cleavage of longer dsRNAs, DICER endonuclease plays important role in the loading of cleaved dsRNA into the RISC complex (Lee et al., 2004; Vergani-Junior et al., 2021). Thus, the presence of DICER enzyme sites is desirable when selecting target regions for dsRNA design. Another interesting optimization of dsRNA molecule aiming at enhancing dsRNA activity for exogenously applied treatments to plants and insect ingestion was demonstrated by Hunter and Wintermantel (Hunter et al., 2021). Authors reported that chemically-modified dsRNAs incorporating 2′-F pyrimidine nucleotides (32–55%) along with dsRNA structure, led to considerable improvements in the RNAi activity across multiple Hemipteran insect plant-disease vectors which reflected in increased insect mortality by 12–35% greater than non-modified dsRNAs displaying the same sequence.

Fortunately, the availability of stringent software to design dsRNAs has largely minimized the occurrence of off-target and nontarget effects by predicting the degree of sequence homology between the antisense strand of siRNAs and target transcripts (Knott et al., 2014; Lück et al., 2019). However, for species lacking genome/transcriptome sequence annotation on databases, such bioinformatic-based dsRNA design may require alternative tools and even more important, supplemental information about the biology of target organisms and the existing ecological interactions, in which the dsRNA will be applied (Fletcher et al., 2020).

3.3.2 dsRNA Association With Nanomaterials

A promising alternative to circumvent all aforementioned constraints mainly related to dsRNA uptake, delivery, and stability, boosting the practical use of topical RNAi-based technologies, is the association with nanobiotechnology. The

nanomaterial can be engineered to synthesize NP that operates as nanocarriers for the delivery of dsRNAs, providing several advantages, including protection/stability enhancement of dsRNA molecules, improvement of foliar/microorganism surface adherence, and cell internalization, with positive impacts on the efficacy of RNAi gene silence response (Ghormade et al., 2011; Adeyinka et al., 2020). There is an ever-expanding list of NPs that have already been tested as dsRNA carriers, and they are usually made from lipid biomolecules or different polymers, which can be natural (e.g., agar, starches, alginates, chitosan, and cellulose), synthetics [e.g. poly(vinyl alcohol)—PVA, poly(ethylene glycol)—PEG, and poly(lactic-co-glycolic acid)—PLGA] or hybrids (Sikder et al., 2021). The major challenge in elaborating these NPs lies in the fact that they need to be quite stable, non-toxic, eco-friendly (e.g., biodegradable), and easy to be conjugated with RNAs molecules. Moreover, there are several relevant characteristics of the NPs to be taken into account for the efficient delivery of dsRNAs. For example, in theory, particles larger than 5–20 nm are not capable of entering the plant cell wall (Schwab et al., 2016). Likewise, NPs must be designed to carry positive amino groups to allow the binding with the negatively charged dsRNAs phosphate groups (Avila et al., 2018). Lastly, the complex NP-dsRNA must be able to dissociate into the cell cytosol, and the addition of polyanions molecules or acid solution can confer such ability (Yan et al., 2021).

Among the dsRNA nanoformulations, lipid-based NP (e.g., liposomes and micelles) and chitosan-based dsRNA formulations are by far the most widely used nanocarriers. Numerous studies mostly involving insect species (e.g., *Aedes aegypti*, *Blattella germanica*, *Chilo suppressalis*, *D. melanogaster*, *Euschistus heros*, *Ostrinia nubilalis,* and *Spodoptera frugiperda*), have reported success using these NPs carrying small RNAs to knockdown different gene targets, showing as well an enhancement of dsRNA stability in the presence insect endonuclease enzymes (Wang K. et al., 2020; Christiaens et al., 2020; Gurusamy et al., 2020; Cooper et al., 2021). However, although the high efficiency of lipid-based vesicles in the control of plant pathogens/pests, its practical usage is majorly halted by the high cost and dependence of adjuvants (e.g., surfactant, emulsifier, and stabilizer) used on the generation process (Bauer et al., 2006; Azarnezhad et al., 2020). Nevertheless, several other innovative NPs have been created, expanding dsRNA delivery strategies. Mitter et al. (Mitter et al., 2017) complexed dsRNA with LDH nanosheets, termed Bioclay, which allowed to expand the window of protection from viral pathogens from 5 to 7 days to more than 20 days. Another formulation complexing NP-dsRNA-adjuvants was able to penetrate through the aphid body wall into the haemocoel and spread into various tissues, resulting in significant knockdown of target gene expression and insect mortality (Zheng et al., 2019). Even in recalcitrant insects such Lepidoptera, dsRNA complexed with a synthetic cationic polymer, poly-[N-(3-guanidinopropyl)-methacrylamide], was effectively taken up by *S. frugiperda*, resulting in significant knockdown and larvae mortality (Parsons et al., 2018).

Furthermore, complexing dsRNA molecules to NP hold also the potential to address another big challenge related to the cost of dsRNA synthesis. The production of quantity and quality dsRNA for spray applications is still considered expensive, although the cost (per Gram) to synthesize dsRNA has been considerably reduced, dropping from U$ 12.500 in 2008 to U$ 0.5 in 2021 (Zotti et al., 2018; Rank and Koch, 2021). A low-cost dsRNA production is imperative due to the necessity of applying approximately 2–10 g of dsRNA per hectare (Zotti et al., 2018).

Taken together, technological advances in dsRNA nanoformulations hold the capacity to overcome inherent bottlenecks of topical RNAi-based technique, providing the desirable molecule protection, higher efficiency of dsRNA uptake, and delivery, beyond reducing potential collateral environmental risks. All crucial features for the full establishment of these next-generation crop protection solutions.

4 PUBLIC ACCEPTANCE AND REGULATORY ASPECTS OF CRISPR/CAS AND TOPICAL RNAI-BASED TECHNOLOGIES

The fact that only a handful of these bioproducts and varieties have been approved for commercial release worldwide is probably not only related to the everlasting regulatory hurdles, but also unsettled consumer perception and acceptance (Mat Jalaluddin et al., 2019). According to Taning et al. (Taning et al., 2021b), for society to accept biotechnology products, diverse key tasks should be addressed.

In terms of reporting biotechnology advancements, regular communication among researchers, farmers, and other relevant players in the food production chain are crucial to reassure stakeholders, assist regulatory compliance, and also to support the general public (e.g., consumers) perception. Moreover, the public acceptance of CRISPR/Cas- and RNAi-based bioproducts (e.g., plant crop resistant varieties, biopesticides), mostly relies on a proper and unbiased broadcast addressing technical issues (e.g., gene editing/silencing driving mechanisms), as well as all potential negative and positive (risk-benefits) related impacts. In this process, scientists may play key roles in finding instruments for a straight dialogue with civil society organizations, and supporting educational initiatives (Taning et al., 2021b; Rank and Koch, 2021).

Ethical and moral issues should also be properly addressed early on in the development process of CRISPR/Cas- and RNAi-based technological solutions, since these concepts exert a strong appeal to the target audience (Frewer et al., 2013; Gupta et al., 2015). According to Beghin and Gustafson (Beghin and Gustafson, 2021), most consumers are willing to consume and pay for foods derived from more sustainable plant engineering techniques, especially if they embody useful traits for the environment, animal, and human health. Additional studies have suggested that the use of topical applied RNAi-based products for plant crop disease management may increase public acceptance since this new technology does not involve a

stable expression of transgenic genetic elements by treated organisms (Shew et al., 2017). Similar public behavior was observed for the food consumption of non-transgenic CRISPR/Cas bioproducts already launched (Shew et al., 2018). In these two last-mentioned studies, the authors aimed to test the market viability of RNAi- and CRISPR-based bioproducts, respectively. For this purpose, consumers from different countries, including the United States, Canada, Australia, France, and Belgium, were surveyed for their preference for consuming three bioproducts: a hypothetical GMO rice variety developed by using *Bacillus thuringiensis* (Bt) transgene technology, a hypothetical non-GMO rice variety generated by SIGS approach (i.e., topical RNAi-based technology), and a CRISPR-based crop. The results showed that applicants from all countries were far more inclined to consume non-GMO rice. In addition, authors reported that on average, half of the participants would consume both GMO and CRISPR food. Further studies and more exhaustive field surveys are very welcomed to endorse public acceptance and perception of these new agricultural technologies. Ultimately, to assure a sustainable production of high-quality food, the entire production chain must be ruled with parsimony and balance between environmental, economic, and social claims, as well as be assisted ideally by strong public policies that safeguard consumers' health and their concerns (Montenegro, 2016; Hamburger, 2018).

Concerning the regulatory aspects of CRISPR edited plants, even though the discussion is still ongoing worldwide, several countries already have specific regulatory policies for evaluating these products. Technologies generated through gene editing can be classified as SDN1, SDN2, and SDN3 (SDN, site-directed nucleases) following the terminology proposed by Podevin et al. (Podevin et al., 2013). Regarding the SDN1 strategy, the non-homologous end joining (NHEJ) cell repair pathway is explored mainly to induce gene knockout. In the case of SDN2, the homology-directed repair (HDR) pathway is used to introduce mutations resulting in the alteration of one or few base pairs, for example, to make an allelic substitution. In the SDN3, although it explores the same repair pathway as in SDN2, the inserted sequence is longer and could be a promoter, coding, or terminator region, from a sexually compatible species or not (Podevin et al., 2013). Therefore, depending on the strategy employed, it could or not generate a final product ruled as GMO, even though in many cases it does not involve the introduction of exogenous DNA sequences.

The worldwide scenario of regulatory policies for the evaluation of CRISPR edited plants is changing rapidly and continues to evolve as more countries launch their own regulatory policies, an expanding list which includes so far Argentina, Brazil, Chile, Colombia, United States, Paraguay, Japan, Australia and, more recently, the United Kingdom (Entine et al., 2021). The main focus of the deliberations is still on the question of "*be or not to be*" a GMO and, although the criteria adopted by each of these countries are quite different, in most situations the risk assessment is evaluated case-by-case. Such tailored-made assessment takes into account specific parameters, including the CRISPR-toolbox strategy employed for genome editing, the resulting combination of the genetic material, whether the mutation could be generated by conventional breeding or mutagenesis, and the absence of recombinant DNA in the final product (Molinari et al., 2021).

Briefly, according to the aforementioned legislation, mutations produced by SDN1 systems generate products not qualified as GMOs, and for this reason, they are not evaluated under the same criteria applied for conventional genetically modified products (Jenkins et al., 2021; Molinari et al., 2021). Technological solutions originating from SDN2 may or may not be ruled as GMO under the legislation of most countries in the Americas, with the analysis made on a case-by-case basis. In addition, the major parameter to classify SDN2-based products as GMOs is the presence of exogenous DNA in the final product. In the case of SDN3 systems, due to the complexity of the genetic elements introduced in the recipient genome, its derived products are frequently qualified as GMOs, although being assessed case-by-case, as well as considering the origin of the DNA used (Molinari et al., 2021).

A different position was adopted by some countries of the European Union and New Zealand. So far, they decide that plants obtained through gene editing will follow the same criteria applied to GMOs, regardless of the genome editing strategy employed (Jenkins et al., 2021). The People's Republic of China, despite its outstanding role in the world trade of commodities, has not yet launched regulatory policies for the evaluation of edited plants. These singularities in terms of legislation between countries seem to be linked with different economic aspects, social practices and behaviors, and also political backgrounds. Nevertheless, non-compatible regulatory processes are problematic for international trades, especially in the case of agricultural commodities (Entine et al., 2021). The scientific community, in general, has argued and supported a global level alignment of regulatory policies, which should preserve and strengthen general biosafety requirements, while converging towards the exclusion of some edited bioproducts from the scope of GMOs, depending on the genome editing strategy used. The main point is that whether the obstacles imposed for risk assessment of the edited products were larger than the risks, it surely will discourage innovation, due to increments of costs and time for the technology commercial release. Moreover, in countries where legislation considers that certain products of gene editing may be excluded from GMOs' scope, there has been a remarkable growth in the number of startups and small and medium-sized biotechnology companies (Entine et al., 2021). Ultimately, it would benefit farmers and final consumers with a wide range of technologies generating superior agronomic traits and better nutritional quality agricultural products.

On the other hand, for the use of topical RNAi-based products in agriculture, worldwide regulatory aspects are still in infancy. In many countries, genetic engineering approaches based on this new technology do not fall within the legislation scope applied to GMOs, nor in the legislation applied to conventional chemical and biological pesticides. Due to its potential advantages, manifold studies on plant protection have been carried out aiming at the development of topically-applied RNAi-based bioproducts.

In 2019, the scientific, industrial and governmental communities gathered at the conference of the Organization for Economic Cooperation and Development (OECD, Paris, France), to discuss various aspects of the technology, and guidelines for risk assessment on human, animal, and environmental health were settled (Mendelsohn et al., 2020). One of the most prominent considerations that emerged from this conference was the strong recommendation to carefully analyze the potential off-targets in the risk assessment of these technologies. The availability of *in silico* tools and the growing genomic data annotation for several species have enabled researchers to identify efficient and specific small RNA molecules, including dsRNAs, reducing the risks of off-targets. This is a clear advantage of that technology over the routinely applied chemical pesticides with a broad spectrum of action, hence, affecting also non-target species (Taning et al., 2020). Moreover, it is recommended that risk analysis likewise look over the lifecycle of RNAi-based products in varied environmental conditions, which in many situations may require reapplication (Mendelsohn et al., 2020).

Pre-existing regulatory frameworks for chemical pesticides and bio-inputs risk assessment in different countries could be used as a basis for evaluating products from RNAi, as long as the specificities of this technology are respected. It is worth noting that if the design and development of these products are performed carefully and rigorously, these technologies might revolutionize with an effective and safe basis to manage pests, weeds, and pathogens effectively.

5 CASE STUDIES AND PROSPECTS ON THE HORIZON

To date, no topical RNAi-based herbicide/pesticide has been used commercially. However, numerous patents involving topical RNAi for use in agriculture have been applied (Mat Jalaluddin et al., 2019), showing the importance of a worldwide definition of the regulation of these technologies.

Actually, there are commercially approved RNAi-based transgenic crops, like the RNAi insecticidal maize, the soybean with improved fatty acid profile, the non-browning Arctic® apple, and the low lignin alfalfa (Mat Jalaluddin et al., 2019). However, the whole process of the development and the commercial approval of genetic engineering plants is slow, costly, and for various species very difficult to achieve. Besides, transgenic plants face various regulatory barriers since the first genetically engineered plant was approved in 1994 (Smyth, 2020). It is expected to achieve endogenous plant gene silence using dsRNA at a low cost when compared to GMOs development (Das and Sherif, 2020). In addition, RNAi-based technology with topically applied dsRNA presents low toxicity, and it is species-specific and designed to minimize off-target impacts. Only closely related species to the target presents more risk to be susceptible due to genetic similarity, whereas risks to human health and the environment are very unlikely (Fletcher et al., 2020).

Advances in exploring the use of RNAi technology for crop protection are enabling research results to be transformed into products that are reaching the market. After the commercial release of the first plant expressing dsRNA for pest control (Smart-Stax PRO "MON87411"), Rodrigues et al. (Rodrigues et al., 2021) announced the application for registration of the first sprayable biopesticide based on dsRNA (Ledprona®) intended for the control of the Colorado potato beetle (*L. decemlineata*). The RNAi-based biopesticide is in the process of being registered by the United States Environmental Protection Agency, and *in vivo* tests showed that the Ledprona has an efficiency similar to the Spinosad® insecticide. In Brazil, the Evolluta Agro Biotecnologia Ltda. intends to launch the product "EVO-201A," based on the dsRNAs topical application to control *S. frugiperda* and *Helicoverpa armigera*. The product has already been classified by the Comissão Técnica Nacional de Biossegurança (CTNBio) as a non-GMO (CTNBio, 2020). As it is a product under development, no data were revealed regarding the method of dsRNA delivery or the efficiency in control, but it represents a great advance for the development of products based on the dsRNA topical application in agriculture. These events have reinforced the discussion about the environmental safety of the technology for this application.

On the other hand, a large number of plants with an edited genome by CRISPR/Cas are released for cultivation all around the world, in particular United States and Canada. Nowadays, 37 genetically edited organisms using CRISPR/Cas technology have been cleared (i.e., designated as non-regulated product) by the U.S. Department of Agriculture's Animal and Plant Health Inspection Service (USDA APHIS), being the vast majority composed of plants (USDA APHIS, 2022). In 2016, The Paris mushroom (*Agaricus bisporus*) was the first organism edited using the technology CRISPR/Cas to be designated by the USDA as non-regulated. This product, which normally displayed the darkening of tissues, after the knockout of the polyphenol oxidase (PPO) gene, showed a reduction in the darkening of tissues by 30% and an increase in its shelf life of mushroom (Waltz, 2016). Days after the edited mushroom being cleared by the USDA, the "waxy corn" cultivar, with starch composed entirely of amylopectin, received the same designation as non-regulated product by USDA (Gao et al., 2019; USDA APHIS, 2022). Waxy corn is extremely important for the food, paper, and adhesives industry in the United States, where 2.1 million tons/year are produced in an area of 202.3 thousand hectares (Gao et al., 2019). These and other edited plants and products have been released to commercialization after being exempt of regulation, such as a soybean with high oil and protein content; corn edited to increase drought tolerance and yield stability; plants edited for fungal, bacteria, and herbicides resistance as well as a plant with adapted architectures to different cropping systems (Turnbull et al., 2021; USDA APHIS, 2022). It was in Japan that, for the first time, a product with a genome edited by CRISPR/Cas was released for direct consumption—the tomato variety "Sicilian Rouge High GABA." This variety has been available in Japanese supermarkets since September 2021 (Newscientist, 2021). Tomato plants naturally contain high levels of gamma-aminobutyric acid (GABA), a beneficial amino acid used for the treatment and prevention of chronic disease that affects the

population. The knockout of an auto-inhibitory domain that regulates enzymatic glutamate decarboxylase (GAD) using CRISPR-Cas technology, specifically the knockout of SlGAD2 and SlGAD3 genes, resulting in the occurrence of plants with a 5- to 7-fold greater capacity to produce GABA (Nonaka et al., 2017). The "Sicilian Rouge High GABA" tomato variety is one of the few products already available for consumption and represents an easy and realistic way for consumers to improve their daily diet.

In Brazil, the first plant edited by CRISPR/Cas released for cultivation was the waxy corn from Corteva in 2018. Furthermore, Brazilian researchers developed (through DNA-transgene free CRISPR genome editing) the first non-GMO sugarcane in the world to be considered as non-GMO on 10th December 2021, according to the CTNBio—Normative Resolution 16 (RN 16) (CTNBio, 2018). The sugarcane varieties (Flex and Flex II) offer higher cell wall digestibility and higher sucrose content in plant tissues, respectively. More recently, the soybean edited with low raffinose was also considered as non-GMO by CTNBio on 9th March 2022. Also in South America, a non-GMO potato with reduced enzymatic browning was obtained by the knock-out of a tuber specific polyphenol oxidase (González et al., 2021) (Res. NO-2020-65450768-APN-SABYDR#MAGYP). This variety is under field trials for cultivar registration as a conventional breeding product in Argentina.

Finally, the long-lasting coupling between scientific research and biotechnology has been leading to unprecedented improvements in agricultural products, represented in this review mainly by plant crops, commodities responsible for feeding the globe. CRISPR/Cas- and RNAi-based technologies have revolutionized science and biotechnology due to their high precision, versatility, and relative ease of use, with factual agricultural bioproducts already on the market shelves. Beyond gains in productivity and profitability, these new cultivars (adapted to a broader range of adverse conditions, resistant to diseases and herbicides), eco-friendly bio-pesticides, and all derivative biotechnological solutions hold great potential to solve critical agriculture and environmental issues worldwide, likewise ensuring a sustainable global food supply. However, as reasoned throughout the manuscript, there are still crucial challenges (e.g., delivery, uptake, and stability of the components) and relevant safety issues (e.g., off-/non-target effects and toxicity) to be addressed for a full bench-to-field biotechnological transition. Fortunately, the remarkable fast-paced expansion of both technologies generates an ambience of permanent improvement, which positively impacts the developmental progress of these next-generation crop protection bioproducts. Furthermore, manifold research groups highlight the key role of nanotechnology in the creation of transformational tools suitable to overcome most of the above-mentioned challenges, faced by CRISPR- and topical RNAi-based solutions. Although fine-tune adjustments are still required to overcome inherent technical bottlenecks, it seems that the greatest challenge to be faced towards the full usage of those technologies in modern agriculture is linked with social and political matters (Sprink et al., 2016). Nevertheless, the scientific community, through its inherent transparency and commitment, play key role in the desired convergence of global regulatory landscapes, also in supporting public perception and trust, translating into positive impacts in regulatory policy approvals related to agricultural bioproducts (Maximiano et al., 2021).

AUTHOR CONTRIBUTIONS

FT, FdeA, CdeM, NC, FB, EC, LF, KO, NL, TC, LM, PA, SF, WH, MF, AK, AL, TS, and HC wrote the manuscript. FT organized and revised the final manuscript and created the figure. TS, and HC conceived the review and revised the final manuscript. All authors contributed to this review article and approved the submitted version.

FUNDING

This review was supported by grants from the Fundação de Apoio à Pesquisa do Distrito Federal (Project 00193-00000783/2021-16), Conselho Nacional de Desenvolvimento Científico e Tecnológico (Project 421810/2021-1), and from Embrapa (Projects SEG 20.18.02.001.00.00 and SEG 30.20.00.068.00.00).

REFERENCES

Adeyinka, O. S., Riaz, S., Toufiq, N., Yousaf, I., Bhatti, M. U., and Batcho, A. (2020). Advances in Exogenous RNA Delivery Techniques for RNAi-Mediated Pest Control. *Mol. Biol. Rep.* 47, 6309–6319. doi:10.1007/S11033-020-05666-2/FIGURES/1

Ali, Z., Abul-Faraj, A., Li, L., Ghosh, N., Piatek, M., Mahjoub, A., et al. (2015a). Efficient Virus-Mediated Genome Editing in Plants Using the CRISPR/Cas9 System. *Mol. Plant* 8, 1288–1291. doi:10.1016/J.MOLP.2015.02.011

Ali, Z., Abulfaraj, A., Idris, A., Ali, S., Tashkandi, M., and Mahfouz, M. M. (2015b). CRISPR/Cas9-mediated Viral Interference in Plants. *Genome Biol.* 16, 238. doi:10.1186/s13059-015-0799-6

Altpeter, F., Springer, N. M., Bartley, L. E., Blechl, A. E., Brutnell, T. P., Citovsky, V., et al. (2016). Advancing Crop Transformation in the Era of Genome Editing. *Plant Cell.* 28, 1510–1520. doi:10.1105/tpc.16.00196

Andersson, M., Turesson, H., Olsson, N., Fält, A. S., Ohlsson, P., Gonzalez, M. N., et al. (2018). Genome Editing in Potato via CRISPR-Cas9 Ribonucleoprotein Delivery. *Physiol. Plant.* 164, 378–384. doi:10.1111/PPL.12731

Anzalone, A. V., Koblan, L. W., and Liu, D. R. (2020). Genome Editing with CRISPR–Cas Nucleases, Base Editors, Transposases and Prime Editors. *Nat. Biotechnol.* 387 38, 824–844. doi:10.1038/s41587-020-0561-9

Aronstein, K., Pankiw, T., and Saldivar, E. (2015). SID-I Is Implicated in Systemic Gene Silencing in the Honey Bee 45, 20–24. doi:10.1080/00218839.2006.11101307

Arpaia, S., Christiaens, O., Giddings, K., Jones, H., Mezzetti, B., Moronta-Barrios, F., et al. (2020). Biosafety of GM Crop Plants Expressing dsRNA: Data Requirements and EU Regulatory Considerations. *Front. Plant Sci.* 11, 940. doi:10.3389/FPLS.2020.00940/BIBTEX

Avila, L. A., Chandrasekar, R., Wilkinson, K. E., Balthazor, J., Heerman, M., Bechard, J., et al. (2018). Delivery of Lethal dsRNAs in Insect Diets by Branched Amphiphilic Peptide Capsules. *J. Control. Release* 273, 139–146. doi:10.1016/J.JCONREL.2018.01.010

Azarnezhad, A., Samadian, H., Jaymand, M., Sobhani, M., and Ahmadi, A. (2020). Toxicological Profile of Lipid-Based Nanostructures: Are They Considered as Completely Safe Nanocarriers? *Crit. Rev. Toxicol.* 50, 148–176. doi:10.1080/10408444.2020.1719974

Bachman, P., Fischer, J., Song, Z., Urbanczyk-Wochniak, E., and Watson, G. (2020). Environmental Fate and Dissipation of Applied dsRNA in Soil, Aquatic Systems, and Plants. *Front. Plant Sci.* 11. doi:10.3389/FPLS.2020.00021

Bae, S., Park, J., and Kim, J. S. (2014). Cas-OFFinder: A Fast and Versatile Algorithm that Searches for Potential Off-Target Sites of Cas9 RNA-Guided Endonucleases. *Bioinformatics* 30, 1473–1475. doi:10.1093/bioinformatics/btu048

Baldrich, P., Rutter, B. D., Karimi, H. Z., Podicheti, R., Meyers, B. C., and Innes, R. W. b. (2019). Plant Extracellular Vesicles Contain Diverse Small RNA Species and Are Enriched in 10- to 17-Nucleotide "Tiny" RNAs. *Plant Cell.* 31, 315–324. doi:10.1105/TPC.18.00872

Bansal, R., and Michel, A. P. (2013). Core RNAi Machinery and Sid1, a Component for Systemic RNAi, in the Hemipteran Insect, Aphis Glycines. *Int. J. Mol. Sci.* 14, 3786–3801. doi:10.3390/IJMS14023786

Bao, W., Wan, Y., and Baluška, F. (2017). Nanosheets for Delivery of Biomolecules into Plant Cells. *Trends Plant Sci.* 22, 445–447. doi:10.1016/j.tplants.2017.03.014

Bauer, M., Kristensen, B. W., Meyer, M., Gasser, T., Widmer, H. R., Zimmer, J., et al. (2006). Toxic Effects of Lipid-Mediated Gene Transfer in Ventral Mesencephalic Explant Cultures. *Basic Clin. Pharmacol. Toxicol.* 98, 395–400. doi:10.1111/J.1742-7843.2006.PTO_310.X

Baum, J. a., Bogaert, T., Clinton, W., Heck, G. R., Feldmann, P., Ilagan, O., et al. (2007). Control of Coleopteran Insect Pests through RNA Interference. *Nat. Biotechnol.* 25, 1322–1326. doi:10.1038/nbt1359

Beghin, J. C., and Gustafson, C. R. (2021). Consumer Valuation of and Attitudes towards Novel Foods Produced with New Plant Engineering Techniques: A Review. *Sustain* 202113, 11348. doi:10.3390/SU132011348

Bennett, M., Deikman, J., Hendrix, B., and Iandolino, A. (2020). Barriers to Efficient Foliar Uptake of dsRNA and Molecular Barriers to dsRNA Activity in Plant Cells. *Front. Plant Sci.* 11, 816. doi:10.3389/FPLS.2020.00816/BIBTEX

Bleackley, M. R., Samuel, M., Garcia-Ceron, D., McKenna, J. A., Lowe, R. G. T., Pathan, M., et al. (2020). Extracellular Vesicles from the Cotton Pathogen Fusarium Oxysporum F. Sp. Vasinfectum Induce a Phytotoxic Response in Plants. *Front. Plant Sci.* 10, 1610. doi:10.3389/FPLS.2019.01610/BIBTEX

Bortesi, L., and Fischer, R. (2015). The CRISPR/Cas9 System for Plant Genome Editing and beyond. *Biotechnol. Adv.* 33, 41–52. doi:10.1016/j.biotechadv.2014.12.006

Bossdorf, O., Arcuri, D., Richards, C. L., and Pigliucci, M. (2010). Experimental Alteration of DNA Methylation Affects the Phenotypic Plasticity of Ecologically Relevant Traits in *Arabidopsis thaliana*. *Evol. Ecol.* 24, 541–553. doi:10.1007/S10682-010-9372-7/TABLES/4

Budzinski, H., and Couderchet, M. (2018). Environmental and Human Health Issues Related to Pesticides: from Usage and Environmental Fate to Impact. *Environ. Sci. Pollut. Res.* 2515 25, 14277–14279. doi:10.1007/S11356-018-1738-3

Cai, Q., Qiao, L., Wang, M., He, B., Lin, F. M., Palmquist, J., et al. (2018). Plants Send Small RNAs in Extracellular Vesicles to Fungal Pathogen to Silence Virulence Genes. *Science* 360, 1126–1129. doi:10.1126/SCIENCE.AAR4142

Cameron, P., Cameron, P., Settle, A. H., Fuller, C. K., Thompson, M. S., Cigan, A. M., et al. (2017). SITE-seq: A Genome-wide Method to Measure Cas9 Cleavage. *Protoc. Exch.* 4–13. doi:10.1038/protex.2017.043

Cappelle, K., De Oliveira, C. F. R., Van Eynde, B., Christiaens, O., and Smagghe, G. (2016). The Involvement of Clathrin-Mediated Endocytosis and Two Sid-1-like Transmembrane Proteins in Double-Stranded RNA Uptake in the Colorado Potato Beetle Midgut. *Insect Mol. Biol.* 25, 315–323. doi:10.1111/IMB.12222

Chen, J., Peng, Y., Zhang, H., Wang, K., Zhao, C., Zhu, G., et al. (2021a). Off-target Effects of RNAi Correlate with the Mismatch Rate between dsRNA and Non-target mRNA. *RNA Biol.* 18, 1747. doi:10.1080/15476286.2020.1868680

Chen, J., Peng, Y., Zhang, H., Wang, K., Zhao, C., Zhu, G., et al. (2021b). Off-target Effects of RNAi Correlate with the Mismatch Rate between dsRNA and Non-target mRNA. *RNA Biol.* 18, 1747. doi:10.1080/15476286.2020.1868680

Cho, S., Choe, D., Lee, E., Kim, S. C., Palsson, B., and Cho, B. K. (2018). High-Level dCas9 Expression Induces Abnormal Cell Morphology in *Escherichia coli*. *ACS Synth. Biol.* 7, 1085–1094. doi:10.1021/ACSSYNBIO.7B00462/SUPPL_FILE/SB7B00462_SI_005.XLSX

Christiaens, O., Petek, M., Smagghe, G., and Taning, C. N. T. (2020). The Use of Nanocarriers to Improve the Efficiency of RNAi-Based Pesticides in Agriculture. *Nanopesticides*, 49–68. doi:10.1007/978-3-030-44873-8_3

Cooper, A. M. W., Song, H., Yu, Z., Biondi, M., Bai, J., Shi, X., et al. (2021). Comparison of Strategies for Enhancing RNA Interference Efficiency in Ostrinia Nubilalis. *Pest Manag. Sci.* 77, 635–645. doi:10.1002/PS.6114

Cunningham, F. J., Goh, N. S., Demirer, G. S., Matos, J. L., and Landry, M. P. (2018). Nanoparticle-Mediated Delivery towards Advancing Plant Genetic Engineering. *Trends Biotechnol.* 36, 882–897. doi:10.1016/j.tibtech.2018.03.009

Dalakouras, A., and Papadopoulou, K. K. (2020). *Epigenetic Modifications: An Unexplored Facet of Exogenous RNA Application in Plants*. Basel, Switzerland: Plants, 9. doi:10.3390/PLANTS9060673

Dalakouras, A., Wassenegger, M., Dadami, E., Ganopoulos, I., Pappas, M. L., and Papadopoulou, K. (2020). Genetically Modified Organism-free RNA Interference: Exogenous Application of RNA Molecules in Plants1[open]. *Plant Physiol.* 182, 38–50. doi:10.1104/PP.19.00570

Das, A., Ghana, P., Rudrappa, B., Gandhi, R., Tavva, V. S., and Mohanty, A. (2021). Genome Editing of Rice by CRISPR-Cas: End-To-End Pipeline for Crop Improvement. *Methods Mol. Biol.* 2238, 115–134. doi:10.1007/978-1-0716-1068-8_8

Das, P. R., and Sherif, S. M. (2020). Application of Exogenous dsRNAs-Induced RNAi in Agriculture: Challenges and Triumphs. *Front. Plant Sci.* 11. doi:10.3389/FPLS.2020.00946

Demirer, G. S., Silva, T. N., Jackson, C. T., Thomas, J. B., Ehrhardt, W., Rhee, S. Y., et al. (2021). Nanotechnology to Advance CRISPR–Cas Genetic Engineering of Plants. *Nat. Nanotechnol.* 163 (16), 243–250. doi:10.1038/s41565-021-00854-y

Demirer, G. S., Zhang, H., Goh, N. S., Pinals, R. L., Chang, R., and Landry, M. P. (2020). Carbon Nanocarriers Deliver siRNA to Intact Plant Cells for Efficient Gene Knockdown. *Sci. Adv.* 6. doi:10.1126/SCIADV.AAZ0495

Dey, A. (2021). CRISPR/Cas Genome Editing to Optimize Pharmacologically Active Plant Natural Products. *Pharmacol. Res.* 164. doi:10.1016/J.PHRS.2020.105359

Doebley, J. F., Gaut, B. S., and Smith, B. D. (2006). The Molecular Genetics of Crop Domestication. *Cell*. doi:10.1016.j.cell.2006.12.00610.1016/j.cell.2006.12.006

Doench, J. G., Petersen, C. P., and Sharp, P. A. (2003). siRNAs Can Function as miRNAs. *Genes. Dev.* 17, 438–442. doi:10.1101/GAD.1064703

Doyle, C., Higginbottom, K., Swift, T. A., Winfield, M., Bellas, C., Benito-Alifonso, D., et al. (2019). A Simple Method for Spray-On Gene Editing in Planta. *bioRxiv*, 805036. doi:10.1101/805036

Dubrovina, A. S., and Kiselev, K. V. (2019). Exogenous RNAs for Gene Regulation and Plant Resistance. *Int. J. Mol. Sci.* 20. doi:10.3390/IJMS20092282

El-Mounadi, K., Morales-Floriano, M. L., and Garcia-Ruiz, H. (2020). Principles, Applications, and Biosafety of Plant Genome Editing Using CRISPR-Cas9. *Front. Plant Sci.* 11. doi:10.3389/FPLS.2020.00056

Ellison, E. E., Chamness, J. C., and Voytas, D. F. (2021). *Viruses as Vectors for the Delivery of Gene-Editing Reagents*, 97–122. doi:10.19103/as.2020.0082.2810.1201/9781003048237-5

Entine, J., Felipe, M. S. S., Groenewald, J. H., Kershen, D. L., Lema, M., McHughen, A., et al. (2021). Regulatory Approaches for Genome Edited Agricultural Plants in Select Countries and Jurisdictions Around the World. *Transgenic Res.* 30, 551–584. doi:10.1007/S11248-021-00257-8/FIGURES/1

Feng, Z., Mao, Y., Xu, N., Zhang, B., Wei, P., Yang, D. L., et al. (2014). Multigeneration Analysis Reveals the Inheritance, Specificity, and Patterns of CRISPR/Cas-induced Gene Modifications in Arabidopsis. *Proc. Natl. Acad. Sci. U. S. A.* 111, 4632–4637. doi:10.1073/PNAS.1400822111/SUPPL_FILE/SAPP.PDF

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998). Potent and Specific Genetic Interference by Double-Stranded RNA in *Caenorhabditis elegans*. *Nat* 391, 806–811. doi:10.1038/35888

Fletcher, S. J., Reeves, P. T., Hoang, B. T., and Mitter, N. (2020). A Perspective on RNAi-Based Biopesticides. *Front. Plant Sci.* 11, 51. doi:10.3389/FPLS.2020.00051/BIBTEX

Frewer, L. J., van der Lans, I. A., Fischer, A. R. H., Reinders, M. J., Menozzi, D., Zhang, X., et al. (2013). Public Perceptions of Agri-Food Applications of Genetic Modification – A Systematic Review and Meta-Analysis. *Trends Food Sci. Technol.* 30, 142–152. doi:10.1016/J.TIFS.2013.01.003

Fu, Y., Foden, J. A., Khayter, C., Maeder, M. L., Reyon, D., Joung, J. K., et al. (2013). High-frequency Off-Target Mutagenesis Induced by CRISPR-Cas Nucleases in Human Cells. *Nat. Biotechnol.* 319 31, 822–826. doi:10.1038/nbt.2623

Gao, Q., Xu, W. Y., Yan, T., Fang, X. D., Cao, Q., Zhang, Z. J., et al. (2019). Rescue of a Plant Cytorhabdovirus as Versatile Expression Platforms for Planthopper and Cereal Genomic Studies. *New Phytol.* 223, 2120–2133. doi:10.1111/NPH.15889

Gentzel, I. N., Ohlson, E. W., Redinbaugh, M. G., and Wang, G.-L. (2022). VIGE: Virus-Induced Genome Editing for Improving Abiotic and Biotic Stress Traits in Plants. *Stress Biol.*, 1. doi:10.1007/S44154-021-00026-X

Gerashchenkova, G. A., Rozhnova, N. A., Kuluev, B. R., Kiryanova, O. Yu., Gumerova, G. R., Knyazev, A. V., et al. (2020). Design of Guide RNA for CRISPR/Cas Plant Genome Editing. *Mol. Biol.* 54, 24–42. doi:10.1134/S0026893320010069

Ghag, S. B. (2017). Host Induced Gene Silencing, an Emerging Science to Engineer Crop Resistance against Harmful Plant Pathogens. *Physiol. Mol. Plant Pathol.* 100, 242–254. doi:10.1016/J.PMPP.2017.10.003

Ghormade, V., Deshpande, M. V., and Paknikar, K. M. (2011). Perspectives for Nano-Biotechnology Enabled Protection and Nutrition of Plants. *Biotechnol. Adv.* 29, 792–803. doi:10.1016/J.BIOTECHADV.2011.06.007

González, M. N., Massa, G. A., Andersson, M., Décima Oneto, C. A., Turesson, H., Storani, L., et al. (2021). Comparative Potato Genome Editing: Agrobacterium Tumefaciens-Mediated Transformation and Protoplasts Transfection Delivery of CRISPR/Cas9 Components Directed to StPPO2 Gene. *Plant Cell. Tiss. Organ Cult.* 145 (2), 291–305. doi:10.1007/s11240-020-02008-9

Graham, N., Patil, G. B., Bubeck, D. M., Dobert, R. C., Glenn, K. C., Gutsche, A. T., et al. (2020). Plant Genome Editing and the Relevance of Off-Target Changes. *Plant Physiol.* 183, 1453–1471. doi:10.1104/PP.19.01194

Guo, Q., Liu, Q., Smith, N. A., Liang, G., and Wang, M.-B. (2016). RNA Silencing in Plants: Mechanisms, Technologies and Applications in Horticultural Crops. *Curr. Genomics* 17, 476. doi:10.2174/1389202917666160520103117

Guo, Z., Li, Y., and Ding, S. W. (2018). Small RNA-Based Antimicrobial Immunity. *Nat. Rev. Immunol.* 191 19, 31–44. doi:10.1038/s41577-018-0071-x

Gupta, N., Fischer, A. R. H., and Frewer, L. J. (2015). Ethics, Risk and Benefits Associated with Different Applications of Nanotechnology: a Comparison of Expert and Consumer Perceptions of Drivers of Societal Acceptance. *Nanoethics* 9, 93–108. doi:10.1007/S11569-015-0222-5

Gurusamy, D., Mogilicherla, K., Shukla, J. N., and Palli, S. R. (2020). Lipids Help Double-Stranded RNA in Endosomal Escape and Improve RNA Interference in the Fall Armyworm, Spodoptera Frugiperda. *Arch. Insect Biochem. Physiol.* 104, e21678. doi:10.1002/ARCH.21678

Haeussler, M., Schönig, K., Eckert, H., Eschstruth, A., Mianné, J., Renaud, J. B., et al. (2016). Evaluation of Off-Target and On-Target Scoring Algorithms and Integration into the Guide RNA Selection Tool CRISPOR. *Genome Biol.* 17, 1–12. doi:10.1186/s13059-016-1012-2

Hahn, F., and Nekrasov, V. (2019). CRISPR/Cas Precision: Do We Need to Worry about Off-Targeting in Plants? *Plant Cell. Rep.* 38, 437–441. doi:10.1007/S00299-018-2355-9

Hajiahmadi, Z., Movahedi, A., Wei, H., Li, D., Orooji, Y., Ruan, H., et al. (2019). Strategies to Increase On-Target and Reduce Off-Target Effects of the CRISPR/Cas9 System in Plants. *Int. J. Mol. Sci.* 20. doi:10.3390/IJMS20153719

Hamburger, D. J. S. (2018). Normative Criteria and Their Inclusion in a Regulatory Framework for New Plant Varieties Derived from Genome Editing. *Front. Bioeng. Biotechnol.* 6. doi:10.3389/FBIOE.2018.00176

Hashimoto, M., Yamashita, Y., and Takemoto, T. (2016). Electroporation of Cas9 protein/sgRNA into Early Pronuclear Zygotes Generates Non-mosaic Mutants in the Mouse. *Dev. Biol.* 418, 1–9. doi:10.1016/J.YDBIO.2016.07.017

He, Y., and Zhao, Y. (2019). Technological Breakthroughs in Generating Transgene-free and Genetically Stable CRISPR-Edited Plants. *aBIOTECH* 11 (1), 88–96. doi:10.1007/S42994-019-00013-X

Heller, D. A., Jena, P. V., Pasquali, M., Kostarelos, K., Delogu, L. G., Meidl, R. E., et al. (2020). Banning Carbon Nanotubes Would Be Scientifically Unjustified and Damaging to Innovation. *Nat. Nanotechnol.* 153 (15), 164–166. doi:10.1038/s41565-020-0656-y

Hill, E. H., and Solomon, P. S. (2020). Extracellular Vesicles from the Apoplastic Fungal Wheat Pathogen Zymoseptoria Tritici. *Fungal Biol. Biotechnol.* 7, 1–14. doi:10.1186/S40694-020-00103-2/FIGURES/4

Hsu, P. D., Scott, D. A., Weinstein, J. A., Ran, F. A., Konermann, S., Agarwala, V., et al. (2013). DNA Targeting Specificity of RNA-Guided Cas9 Nucleases. *Nat. Biotechnol.* 319 31, 827–832. doi:10.1038/nbt.2647

Huang Holger Puchta, T.-K., Schillberg, S., Huang Á Puchta, T. H., and Orzaez, D. (2021). Novel CRISPR/Cas Applications in Plants: from Prime Editing to Chromosome Engineering. *Transgenic Res.* 304 (30), 529–549. doi:10.1007/S11248-021-00238-X

Hunter, W. B., Cooper, W. R., Sandoval-Mojica, A. F., McCollum, G., Aishwarya, V., and Pelz-Stelinski, K. S. (2021). Improving Suppression of Hemipteran Vectors and Bacterial Pathogens of Citrus and Solanaceous Plants: Advances in Antisense Oligonucleotides (FANA). *Front. Agron.* 3, 1–21. doi:10.3389/fagro.2021.675247

Hunter, W. B., Glick, E., Paldi, N., and Bextine, B. R. (2012). *Advances in RNA Interference: dsRNA Treatment in Trees and Grapevines for Insect Pest Suppression*, 85–87. doi:10.3958/059.037.0110

Ihry, R. J., Worringer, K. A., Salick, M. R., Frias, E., Ho, D., Theriault, K., et al. (2018). p53 Inhibits CRISPR–Cas9 Engineering in Human Pluripotent Stem Cells. *Nat. Med.* 247 24, 939–946. doi:10.1038/s41591-018-0050-6

Imai, R., Hamada, H., Liu, Y., Linghu, Q., Kumagai, Y., Nagira, Y., et al. (2020). In Planta Particle Bombardment (iPB): A New Method for Plant Transformation and Genome Editing. *Plant Biotechnol.* 37, 171. doi:10.5511/PLANTBIOTECHNOLOGY.20.0206A

Jackson, A. L., Bartz, S. R., Schelter, J., Kobayashi, S. V., Burchard, J., Mao, M., et al. (2003). Expression Profiling Reveals Off-Target Gene Regulation by RNAi. *Nat. Biotechnol.* 216 21, 635–637. doi:10.1038/nbt831

Jaenisch, R., and Bird, A. (2003). Epigenetic Regulation of Gene Expression: How the Genome Integrates Intrinsic and Environmental Signals. *Nat. Genet.* 333 33, 245–254. doi:10.1038/ng1089

Jenkins, D., Dobert, R., Atanassova, A., and Pavely, C. (2021). Impacts of the Regulatory Environment for Gene Editing on Delivering Beneficial Products. *Vitro Cell. Dev. Biol. Plant* 57, 609–626. doi:10.1007/S11627-021-10201-4

Jensen, K. T., Fløe, L., Petersen, T. S., Huang, J., Xu, F., Bolund, L., et al. (2017). Chromatin Accessibility and Guide Sequence Secondary Structure Affect CRISPR-Cas9 Gene Editing Efficiency. *FEBS Lett.* 591, 1892–1901. doi:10.1002/1873-3468.12707

Jiang, W., Brueggeman, A. J., Horken, K. M., Plucinak, T. M., and Weeks, D. P. (2014). Successful Transient Expression of Cas9 and Single Guide RNA Genes in Chlamydomonas Reinhardtii. *Eukaryot. Cell.* 13, 1465–1469. doi:10.1128/EC.00213-14

Jiang, Y., Qian, F., Yang, J., Liu, Y., Dong, F., Xu, C., et al. (2017). CRISPR-Cpf1 Assisted Genome Editing of Corynebacterium Glutamicum. *Nat. Commun.* 81 8, 1–11. doi:10.1038/ncomms15179

Jin, S., Lin, Q., Luo, Y., Zhu, Z., Liu, G., Li, Y., et al. (2021). Genome-wide Specificity of Prime Editors in Plants. *Nat. Biotechnol.* 39, 1292–1299. doi:10.1038/s41587-021-00891-x

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., and Charpentier, E. (2012). A Programmable Dual-RNA – Guided 337, 816–822. doi:10.1126/science.1225829

Jorasch, P. (2019). The Global Need for Plant Breeding Innovation. *Transgenic Res.* 28, 81–86. doi:10.1007/S11248-019-00138-1/FIGURES/4

Kale, L., Nakurte, I., Jalakas, P., Kunga-Jegere, L., Brosché, M., and Rostoks, N. (2019). Arabidopsis Mutant Dnd2 Exhibits Increased Auxin and Abscisic Acid Content and Reduced Stomatal Conductance. *Plant Physiol. biochem.* 140, 18–26. doi:10.1016/J.PLAPHY.2019.05.004

Kalyandurg, P. B., Sundararajan, P., Dubey, M., Ghadamgahi, F., Zahid, M. A., Whisson, S. C., et al. (2021). Spray-induced Gene Silencing as a Potential Tool to Control Potato Late Blight Disease. *Phytopathology* 111, 2166–2175. doi:10.1094/PHYTO-02-21-0054-SC

Kaya, H., Mikami, M., Endo, A., Endo, M., and Toki, S. (2016). Highly Specific Targeted Mutagenesis in Plants Using *Staphylococcus aureus* Cas9. *Sci. Rep.* 61 (6), 1–9. doi:10.1038/srep26871

Kieu, N. P., Lenman, M., Wang, E. S., Petersen, B. L., and Andreasson, E. (2021). Mutations Introduced in Susceptibility Genes through CRISPR/Cas9 Genome Editing Confer Increased Late Blight Resistance in Potatoes. *Sci. Rep.* 11, 1–12. doi:10.1038/s41598-021-83972-w

Kim, D., Bae, S., Park, J., Kim, E., Kim, S., Yu, H. R., et al. (2015a). Digenome-seq: Genome-wide Profiling of CRISPR-Cas9 Off-Target Effects in Human Cells. *Nat. Methods* 12, 237–243. doi:10.1038/NMETH.3284

Kim, H., Kim, S. T., Ryu, J., Kang, B. C., Kim, J. S., and Kim, S. G. (2017). CRISPR/Cpf1-mediated DNA-free Plant Genome Editing. *Nat. Commun.* 81 8, 1–7. doi:10.1038/ncomms14406

Kim, J.-S., Cho, S. W., Kim, S.-T., Kim, H., Woo, J. W., Choe, S., et al. (2015b). DNA-free Genome Editing in Plants with Preassembled CRISPR-Cas9 Ribonucleoproteins. *Nat. Biotechnol.* 33, 1162–1164. doi:10.1038/nbt.3389

Kim, K. Do, Kang, Y., and Kim, C. (2020). Application of Genomic Big Data in Plant Breeding:Past, Present, and Future. *Plants (Basel, Switz.* 9, 1–25. doi:10.3390/PLANTS9111454

Kiselev, K. V., Suprun, A. R., Aleynova, O. A., Ogneva, Z. V., Kostetsky, E. Y., and Dubrovina, A. S. (2022). The Specificity of Transgene Suppression in Plants by Exogenous dsRNA. *Plants (Basel, Switz.* 11, 715. doi:10.3390/PLANTS11060715

Knott, S. R. V., Maceli, A. R., Erard, N., Chang, K., Marran, K., Zhou, X., et al. (2014). A Computational Algorithm to Predict shRNA Potency. *Mol. Cell.* 56, 796–807. doi:10.1016/j.molcel.2014.10.025

Koch, A., Biedenkopf, D., Furch, A., Weber, L., Rossbach, O., Abdellatef, E., et al. (2016). An RNAi-Based Control of Fusarium Graminearum Infections through Spraying of Long dsRNAs Involves a Plant Passage and Is Controlled by the Fungal Silencing Machinery. *PLOS Pathog.* 12, e1005901. doi:10.1371/JOURNAL.PPAT.1005901

Koch, A., Kumar, N., Weber, L., Keller, H., Imani, J., and Kogel, K. H. (2013). Host-induced Gene Silencing of Cytochrome P450 Lanosterol C14α-Demethylase-Encoding Genes Confers Strong Resistance to Fusarium Species. *Proc. Natl. Acad. Sci. U. S. A.* 110, 19324–19329. doi:10.1073/PNAS.1306373110

Kostarelos, K. (2008). The Long and Short of Carbon Nanotube Toxicity. *Nat. Biotechnol.* 26, 774–776. doi:10.1038/NBT0708-774

Kulkarni, M. M., Booker, M., Silver, S. J., Friedman, A., Hong, P., Perrimon, N., et al. (2006). Evidence of Off-Target Effects Associated with Long dsRNAs in *Drosophila melanogaster* Cell-Based Assays. *Nat. Methods* 3, 833–838. doi:10.1038/NMETH935

Kwak, S. Y., Lew, T. T. S., Sweeney, C. J., Koman, V. B., Wong, M. H., Bohmert-Tatarev, K., et al. (2019). Chloroplast-selective Gene Delivery and Expression in Planta Using Chitosan-Complexed Single-Walled Carbon Nanotube Carriers. *Nat. Nanotechnol.* 145 (14), 447–455. doi:10.1038/s41565-019-0375-4

Labuhn, M., Adams, F. F., Ng, M., Knoess, S., Schambach, A., Charpentier, E. M., et al. (2018). Refined sgRNA Efficacy Prediction Improves Large- and Small-Scale CRISPR-Cas9 Applications. *Nucleic Acids Res.* 46, 1375–1385. doi:10.1093/NAR/GKX1268

Labun, K., Montague, T. G., Gagnon, J. A., Thyme, S. B., and Valen, E. (2016). CHOPCHOP V2: a Web Tool for the Next Generation of CRISPR Genome Engineering. *Nucleic Acids Res.* 44, W272–W276. doi:10.1093/NAR/GKW398

Laird, P. W. (2010). Principles and Challenges of Genome-wide DNA Methylation Analysis. *Nat. Rev. Genet.* 113 11, 191–203. doi:10.1038/nrg2732

Lawrenson, T., Shorinola, O., Stacey, N., Li, C., Østergaard, L., Patron, N., et al. (2015). Induction of Targeted, Heritable Mutations in Barley and *Brassica oleracea* Using RNA-Guided Cas9 Nuclease. *Genome Biol.* 16, 258. doi:10.1186/s13059-015-0826-7

Lee, J. H., Mazarei, M., Pfotenhauer, A. C., Dorrough, A. B., Poindexter, M. R., Hewezi, T., et al. (2020). Epigenetic Footprints of CRISPR/Cas9-Mediated Genome Editing in Plants. *Front. Plant Sci.* 10, 1720. doi:10.3389/FPLS.2019.01720/BIBTEX

Lee, Y. S., Nakahara, K., Pham, J. W., Kim, K., He, Z., Sontheimer, E. J., et al. (2004). Distinct Roles for Drosophila Dicer-1 and Dicer-2 in the siRNA/miRNA Silencing Pathways. *Cell.* 117, 69–81. doi:10.1016/S0092-8674(04)00261-2

Lei, J., Dai, P., Li, Y., Zhang, W., Zhou, G., Liu, C., et al. (2021). Heritable Gene Editing Using FT Mobile Guide RNAs and DNA Viruses. *Plant Methods* 17, 1–11. doi:10.1186/S13007-021-00719-4/FIGURES/4

Li, C., Psatha, N., Gil, S., Wang, H., Papayannopoulou, T., and Lieber, A. (2018). HDAd5/35++ Adenovirus Vector Expressing Anti-CRISPR Peptides Decreases CRISPR/Cas9 Toxicity in Human Hematopoietic Stem Cells. *Mol. Ther. - Methods Clin. Dev.* 9, 390–401. doi:10.1016/J.OMTM.2018.04.008

Liang, Z., Chen, K., Li, T., Zhang, Y., Wang, Y., Zhao, Q., et al. (2017). Efficient DNA-free Genome Editing of Bread Wheat Using CRISPR/Cas9 Ribonucleoprotein Complexes. *Nat. Commun.* 8, 1–5. doi:10.1038/ncomms14261

Liu, G., Kang, G., Wang, S., Huang, Y., and Cai, Q. (2021). Extracellular Vesicles: Emerging Players in Plant Defense against Pathogens. *Front. Plant Sci.* 12. doi:10.3389/FPLS.2021.757925

Liu, G., Yin, K., Zhang, Q., Gao, C., and Qiu, J. L. (2019). Modulating Chromatin Accessibility by Transactivation and Targeting Proximal dsgRNAs Enhances Cas9 Editing Efficiency *In Vivo*. *Genome Biol.* 20, 1–11. doi:10.1186/S13059-019-1762-8/FIGURES/7

Liu, H., Ding, Y., Zhou, Y., Jin, W., Xie, K., and Chen, L. L. (2017). CRISPR-P 2.0: An Improved CRISPR-Cas9 Tool for Genome Editing in Plants. *Mol. Plant* 10, 530–532. doi:10.1016/J.MOLP.2017.01.003

Louwen, R., Staals, R. H. J., Endtz, H. P., van Baarlen, P., and van der Oost, J. (2014). The Role of CRISPR-Cas Systems in Virulence of Pathogenic Bacteria. *Microbiol. Mol. Biol. Rev.* 78, 74–88. doi:10.1128/MMBR.00039-13

Lowder, L. G., Zhou, J., Zhang, Y., Malzahn, A., Zhong, Z., Hsieh, T. F., et al. (2018). Robust Transcriptional Activation in Plants Using Multiplexed CRISPR-Act2.0 and mTALE-Act Systems. *Mol. Plant* 11, 245–256. doi:10.1016/j.molp.2017.11.010

Lück, S., Kreszies, T., Strickert, M., Schweizer, P., Kuhlmann, M., and Douchkov, D. (2019). siRNA-Finder (Si-Fi) Software for RNAi-Target Design and Off-Target Prediction. *Front. Plant Sci.* 10. doi:10.3389/FPLS.2019.01023/FULL

Ma, X., and Li, Z. (2020). Significantly Improved Recovery of Recombinant Sonchus Yellow Net Rhabdovirus by Expressing the Negative-Strand Genomic RNA. *Viruses*, 12. doi:10.3390/V12121459

Ma, X., Zhang, Q., Zhu, Q., Liu, W., Chen, Y., Qiu, R., et al. (2015). A Robust CRISPR/Cas9 System for Convenient, High-Efficiency Multiplex Genome Editing in Monocot and Dicot Plants. *Mol. Plant* 8, 1274–1284. doi:10.1016/j.molp.2015.04.007

Malik, A., Gul, A., Munir, F., Amir, R., Alipour, H., Babar, M. M., et al. (2021). Evaluating the Cleavage Efficacy of CRISPR-Cas9 sgRNAs Targeting Ineffective Regions of *Arabidopsis thaliana* Genome. *PeerJ* 9. doi:10.7717/PEERJ.11409

Markus, B. M., Bell, G. W., Lorenzi, H. A., and Lourido, S. (2019). Optimizing Systems for Cas9 Expression in *Toxoplasma gondii*. *mSphere. 26* 4 (3), e00386-19. doi:10.1128/mSphere.00386-19

Marraffini, L. A. (2015). CRISPR-cas Immunity in Prokaryotes. *Nat* 526, 55–61. doi:10.1038/nature15386

Mat Jalaluddin, N. S., Othman, R. Y., and Harikrishna, J. A. (2019). Global Trends in Research and Commercialization of Exogenous and Endogenous RNAi Technologies for Crops. *Crit. Rev. Biotechnol.* 39, 67–78. doi:10.1080/07388551.2018.1496064

Matzke, M. A., Primig, M., Trnovsky, J., and Matzke, A. J. M. (1989). Reversible Methylation and Inactivation of Marker Genes in Sequentially Transformed Tobacco Plants. *EMBO J.* 8, 643. doi:10.1002/j.1460-2075.1989.tb03421.x

Maximiano, M. R., Távora, F. T. P. K., Prado, G. S., Dias, S. C., Mehta, A., and Franco, O. L. (2021). CRISPR Genome Editing Technology: A Powerful Tool Applied to Developing Agribusiness. *J. Agric. Food Chem.* 69, 6379–6395. doi:10.1021/ACS.JAFC.1C01062/ASSET/IMAGES/ACS.JAFC.1C01062.SOCIAL.JPEG_V03

Mendelsohn, M. L., Gathmann, A., Kardassi, D., Sachana, M., Hopwood, E. M., Dietz-Pfeilstetter, A., et al. (2020). Summary of Discussions from the 2019 OECD Conference on RNAi Based Pesticides. *Front. Plant Sci.* 11, 740. doi:10.3389/FPLS.2020.00740/BIBTEX

Mezzetti, B., Smagghe, G., Arpaia, S., Christiaens, O., Dietz-Pfeilstetter, A., Jones, H., et al. (2020). RNAi: What Is its Position in Agriculture? *J. Pest Sci.* 93, 1125–1130. doi:10.1007/S10340-020-01238-2/FIGURES/3

Miller, S. C., Miyata, K., Brown, S. J., and Tomoyasu, Y. (2012). Dissecting Systemic RNA Interference in the Red Flour Beetle *Tribolium castaneum*: Parameters Affecting the Efficiency of RNAi. *PLoS One* 7, e47431. doi:10.1371/JOURNAL.PONE.0047431

Minkenberg, B., Zhang, J., Xie, K., and Yang, Y. (2019). CRISPR-PLANT V2: an Online Resource for Highly Specific Guide RNA Spacers Based on Improved Off-Target Analysis. *Plant Biotechnol. J.* 17, 5–8. doi:10.1111/PBI.13025

Misra, C. S., Bindal, G., Sodani, M., Wadhawan, S., Kulkarni, S., Gautam, S., et al. (2019). Determination of Cas9/dCas9 Associated Toxicity in Microbes. *BioRxiv*. doi:10.1101/848135

Mitter, N., Worrall, E. A., Robinson, K. E., Li, P., Jain, R. G., Taochy, C., et al. (2017). Clay Nanosheets for Topical Delivery of RNAi for Sustained Protection against Plant Viruses. *Nat. Plants* 32 (3), 1–10. doi:10.1038/nplants.2016.207

Miyata, K., Ramaseshadri, P., Zhang, Y., Segers, G., Bolognesi, R., and Tomoyasu, Y. (2014). Establishing an *In Vivo* Assay System to Identify Components Involved in Environmental RNA Interference in the Western Corn Rootworm. *PLoS One* 9, e101661. doi:10.1371/JOURNAL.PONE.0101661

Modrzejewski, D., Hartung, F., Lehnert, H., Sprink, T., Kohl, C., Keilwagen, J., et al. (2020). Which Factors Affect the Occurrence of Off-Target Effects Caused by the Use of CRISPR/Cas: A Systematic Review in Plants. *Front. Plant Sci.* 11. doi:10.3389/FPLS.2020.574959

Molinari, H. B., Vieira, L. R., Freitas, N. C., Justen, F., de Jesus, V. M., de Oliveira, B. G., et al. (2021). "Regulatory Framework of Genome Editing in Brazil and Worldwide," in *CRISPR Technology in Plant Genome Editing: Biotechnology Applied to Agriculture*. Editors H. B. C. Molinari, L. R. Vieira, N. V. e Silva, G. S. Prado, and J. H. L. Filho (Brasília, DF: Embrapa, 169–195. Available at: https://www.embrapa.br/busca-de-publicacoes/-/publicacao/1132164/regulatory-framework-of-genome-editing-in-brazil-and-worldwide.

Montenegro, M. (2016). No Title. *Cris. Is Coming to Agric. Big Implic. Food, Farmers, Consum. Nat.*, 5. Available at: https://ensia.com/voices/crispr-is-coming-to-agriculture-with-big-implications-for-food-farmers-consumers-and-nature/.

Moradpour, M., and Abdulah, S. N. A. (2020). CRISPR/dCas9 Platforms in Plants: Strategies and Applications beyond Genome Editing. *Plant Biotechnol. J.* 18, 32–44. doi:10.1111/pbi.13232

Morgan, S. L., Mariano, N. C., Bermudez, A., Arruda, N. L., Wu, F., Luo, Y., et al. (2017). Manipulation of Nuclear Architecture through CRISPR-Mediated Chromosomal Looping. *Nat. Commun.* 81 8, 1–9. doi:10.1038/ncomms15993

Murovec, J., Guček, K., Bohanec, B., Avbelj, M., and Jerala, R. (2018). DNA-free Genome Editing of *Brassica oleracea* and B. Rapa Protoplasts Using CRISPR-Cas9 Ribonucleoprotein Complexes. *Front. Plant Sci.* 9. doi:10.3389/FPLS.2018.01594

Napoli, C., Lemieux, C., and Jorgensen, R. (1990). Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-suppression of Homologous Genes in Trans. *Plant Cell.* 2, 279–289. doi:10.1105/TPC.2.4.279

Negishi, K., Kaya, H., Abe, K., Hara, N., Saika, H., and Toki, S. (2019). An Adenine Base Editor with Expanded Targeting Scope Using SpCas9-NGv1 in Rice. *Plant Biotechnol. J.* 17, 1476–1478. doi:10.1111/PBI.13120

Niu, J., Yang, W. J., Tian, Y., Fan, J. Y., Ye, C., Shang, F., et al. (2019). Topical dsRNA Delivery Induces Gene Silencing and Mortality in the Pea Aphid. *Pest Manag. Sci.* 75, 2873–2881. doi:10.1002/PS.5457

Nonaka, S., Arai, C., Takayama, M., Matsukura, C., and Ezura, H. (2017). Efficient Increase of ɣ-Aminobutyric Acid (GABA) Content in Tomato Fruits by Targeted Mutagenesis. *Sci. Rep.* 7. doi:10.1038/S41598-017-06400-Y

Oh, Y., Kim, H., and Kim, S. G. (2021). Virus-induced Plant Genome Editing. *Curr. Opin. Plant Biol.* 60. doi:10.1016/J.PBI.2020.101992

Papikian, A., Liu, W., Gallego-Bartolomé, J., and Jacobsen, S. E. (2019). Site-specific Manipulation of Arabidopsis Loci Using CRISPR-Cas9 SunTag Systems. *Nat. Commun.* 10, 1–11. doi:10.1038/s41467-019-08736-7

Parsons, K. H., Mondal, M. H., McCormick, C. L., and Flynt, A. S. (2018). Guanidinium-Functionalized Interpolyelectrolyte Complexes Enabling RNAi in Resistant Insect Pests. *Biomacromolecules* 19 (4), 1111–1117. doi:10.1021/acs.biomac.7b01717

Peng, A., Chen, S., Lei, T., Xu, L., He, Y., Wu, L., et al. (2017). Engineering Canker-Resistant Plants through CRISPR/Cas9-targeted Editing of the Susceptibility Gene CsLOB1 Promoter in Citrus. *Plant Biotechnol. J.* 15, 1509–1519. doi:10.1111/PBI.12733

Pereira, T. C. (2016). in *Introdução à técnica de CRISPR*. Editor S. B. de G. Ribeirão Preto. 1st ed. SP: SBG.

Pikula, K., Chaika, V., Zakharenko, A., Markina, Z., Vedyagin, A., Kuznetsov, V., et al. (2020). Comparison of the Level and Mechanisms of Toxicity of Carbon Nanotubes, Carbon Nanofibers, and Silicon Nanotubes in Bioassay with Four Marine Microalgae. *Nanomater. (Basel, Switz.* 10. doi:10.3390/NANO10030485

Podevin, N., Davies, H. V., Hartung, F., Nogué, F., and Casacuberta, J. M. (2013). Site-directed Nucleases: a Paradigm Shift in Predictable, Knowledge-Based Plant Breeding. *Trends Biotechnol.* 31, 375–383. doi:10.1016/J.TIBTECH.2013.03.004

Qaim, M. (2020). Role of New Plant Breeding Technologies for Food Security and Sustainable Agricultural Development. *Appl. Econ. Perspect. Policy* 42, 129–150. doi:10.1002/AEPP.13044

Qin, R., Li, J., Li, H., Zhang, Y., Liu, X., Miao, Y., et al. (2019). Developing a Highly Efficient and Wildly Adaptive CRISPR-SaCas9 Toolset for Plant Genome Editing. *Plant Biotechnol. J.* 17, 706. doi:10.1111/PBI.13047

Rajam, M. V. (2020). RNA Silencing Technology: A Boon for Crop Improvement. *J. Biosci.* 45. doi:10.1007/s12038-020-00082-x

Rank, A. P., and Koch, A. (2021). Lab-to-Field Transition of RNA Spray Applications – How Far Are We? *Front. Plant Sci.* 12, 2243. doi:10.3389/FPLS.2021.755203/BIBTEX

Rato, C., Carvalho, M. F., Azevedo, C., and Oblessuc, P. R. (2021). Genome Editing for Resistance against Plant Pests and Pathogens. *Transgenic Res.* 304 (30), 427–459. doi:10.1007/S11248-021-00262-X

Rego-Machado, C. M., Nakasu, E. Y. T., Silva, J. M. F., Lucinda, N., Nagata, T., and Inoue-Nagata, A. K. (2020). siRNA Biogenesis and Advances in Topically Applied dsRNA for Controlling Virus Infections in Tomato Plants. *Sci. Rep.* 10, 1–13. doi:10.1038/s41598-020-79360-5

Ren, C., Li, H., Liu, Y., Li, S., and Liang, Z. (2022). Highly Efficient Activation of Endogenous Gene in Grape Using CRISPR/dCas9-based Transcriptional Activators. *Hortic. Res.* 9. doi:10.1093/hr/uhab037

Ren, X., Yang, Z., Xu, J., Sun, J., Mao, D., Hu, Y., et al. (2014). Enhanced Specificity and Efficiency of the CRISPR/Cas9 System with Optimized sgRNA Parameters in Drosophila. *Cell. Rep.* 9, 1151–1162. doi:10.1016/J.CELREP.2014.09.044

Ricroch, A. E., and Hénard-Damave, M. C. (2016). Next Biotech Plants: New Traits, Crops, Developers and Technologies for Addressing Global Challenges. *Crit. Rev. Biotechnol.* 36, 675–690. doi:10.3109/07388551.2015.1004521

Rodrigues, T., Sridharan, K., Manley, B., Cunningham, D., and Narva, K. (2021). Development of dsRNA as a Sustainable Bioinsecticide: From Laboratory to Field. *ACS Symp. Ser.* 1390, 65–82. doi:10.1021/BK-2021-1390.CH005

Rodríguez-Leal, D., Lemmon, Z. H., Man, J., Bartlett, M. E., and Lippman, Z. B. (2017). Engineering Quantitative Trait Variation for Crop Improvement by Genome Editing. *Cell.* 171, 470–480. e8. doi:10.1016/J.CELL.2017.08.030

Rosenblum, D., Gutkin, A., Dammes, N., and Peer, D. (2020). Progress and Challenges towards CRISPR/Cas Clinical Translation. *Adv. Drug Deliv. Rev.* 154–155, 176–186. doi:10.1016/J.ADDR.2020.07.004

Rutter, B. D., and Innes, R. W. (2017). Extracellular Vesicles Isolated from the Leaf Apoplast Carry Stress-Response Proteins. *Plant Physiol.* 173, 728–741. doi:10.1104/PP.16.01253

San Miguel, K., and Scott, J. G. (2016). The Next Generation of Insecticides: dsRNA Is Stable as a Foliar-Applied Insecticide. *Pest Manag. Sci.* 72, 801–809. doi:10.1002/PS.4056

Sandhya, D., Jogam, P., Allini, V. R., Abbagani, S., and Alok, A. (2020). The Present and Potential Future Methods for Delivering CRISPR/Cas9 Components in Plants. *J. Genet. Eng. Biotechnol.* 18. doi:10.1186/S43141-020-00036-8

Saurabh, S., Vidyarthi, A. S., and Prasad, D. (2014). RNA Interference: Concept to Reality in Crop Improvement. *Planta* 239, 543–564. doi:10.1007/s00425-013-2019-5

Scheben, A., Wolter, F., Batley, J., Puchta, H., and Edwards, D. (2017). Towards CRISPR/Cas Crops - Bringing Together Genomics and Genome Editing. *New Phytol.* 216, 682–698. doi:10.1111/NPH.14702

Schwab, F., Zhai, G., Kern, M., Turner, A., Schnoor, J. L., and Wiesner, M. R. (2016). Barriers, Pathways and Processes for Uptake, Translocation and Accumulation of Nanomaterials in Plants--Critical Review. *Nanotoxicology* 10, 257–278. doi:10.3109/17435390.2015.1048326

Schwarz, D. S., Hutvágner, G., Du, T., Xu, Z., Aronin, N., and Zamore, P. D. (2003). Asymmetry in the Assembly of the RNAi Enzyme Complex. *Cell.* 115, 199–208. doi:10.1016/S0092-8674(03)00759-1/ATTACHMENT/833A2CD6-6739-4806-9436-EDCC21F51100/MMC2.JPG

Shahriar, S. A., Islam, M. N., Chun, C. N. W., Rahim, M. A., Paul, N. C., Uddain, J., et al. (2021). Control of Plant Viral Diseases by CRISPR/Cas9: Resistance Mechanisms, Strategies and Challenges in Food Crops. *Plants* 202110 (126410), 1264. doi:10.3390/PLANTS10071264, *Page*

Shan, Q., Wang, Y., Li, J., Zhang, Y., Chen, K., Liang, Z., et al. (2013). Targeted Genome Modification of Crop Plants Using a CRISPR-Cas System. *Nat. Biotechnol.* 318 31, 686–688. doi:10.1038/nbt.2650

Shew, A. M., Danforth, D. M., Nalley, L. L., Nayga, R. M., Tsiboe, F., and Dixon, B. L. (2017). New Innovations in Agricultural Biotech: Consumer Acceptance of Topical RNAi in Rice Production. *Food control.* 81, 189–195. doi:10.1016/J.FOODCONT.2017.05.047

Shew, A. M., Nalley, L. L., Snell, H. A., Nayga, R. M., and Dixon, B. L. (2018). CRISPR versus GMOs: Public Acceptance and Valuation. *Glob. Food Sec.* 19, 71–80. doi:10.1016/J.GFS.2018.10.005

Shillito, R. D., Whitt, S., Ross, M., Ghavami, F., De Vleesschauwer, D., D'Halluin, K., et al. (2021). Detection of Genome Edits in Plants—From Editing to Seed.

Vitr. Cell. Dev. Biol. - Plant 57, 595–608. doi:10.1007/S11627-021-10214-Z/TABLES/3

Sikder, A., Pearce, A. K., Parkinson, S. J., Napier, R., and O'Reilly, R. K. (2021). Recent Trends in Advanced Polymer Materials in Agriculture Related Applications. *ACS Appl. Polym. Mat.* 3, 1203–1217. doi:10.1021/ACSAPM.0C00982/ASSET/IMAGES/ACSAPM.0C00982.SOCIAL.JPEG_V03

Smyth, S. J. (2020). Regulatory Barriers to Improving Global Food Security. *Glob. Food Sec.* 26, 100440. doi:10.1016/J.GFS.2020.100440

Steinert, J., Schiml, S., Fauser, F., and Puchta, H. (2015). Highly Efficient Heritable Plant Genome Engineering Using Cas9 Orthologues from Streptococcus Thermophilus and *Staphylococcus aureus*. *Plant J.* 84, 1295–1305. doi:10.1111/TPJ.13078

Strohkendl, I., Saifuddin, F. A., Gibson, B. A., Rosen, M. K., Russell, R., and Finkelstein, I. J. (2021). Inhibition of CRISPR-Cas12a DNA Targeting by Nucleosomes and Chromatin. *Sci. Adv.* 7. doi:10.1126/SCIADV.ABD6030

Subburaj, S., Chung, S. J., Lee, C., Ryu, S. M., Kim, D. H., Kim, J. S., et al. (2016). Site-directed Mutagenesis in Petunia × Hybrida Protoplast System Using Direct Delivery of Purified Recombinant Cas9 Ribonucleoproteins. *Plant Cell. Rep.* 35, 1535–1544. doi:10.1007/S00299-016-1937-7

Sukegawa, S., Saika, H., and Toki, S. (2021). Plant Genome Editing: Ever More Precise and Wide Reaching. *Plant J.* 106, 1208–1218. doi:10.1111/TPJ.15233

Svitashev, S., Young, J. K., Schwartz, C., Gao, H., Falco, S. C., and Cigan, A. M. (2015). Targeted Mutagenesis, Precise Gene Editing, and Site-specific Gene Insertion in Maize Using Cas9 and Guide RNA. *Plant Physiol.* 169, 931–945. doi:10.1104/pp.15.00793

Tang, X., Liu, G., Zhou, J., Ren, Q., You, Q., Tian, L., et al. (2018). A Large-Scale Whole-Genome Sequencing Analysis Reveals Highly Specific Genome Editing by Both Cas9 and Cpf1 (Cas12a) Nucleases in Rice. *Genome Biol.* 19, 1–13. doi:10.1186/S13059-018-1458-5/FIGURES/5

Taning, C. N. T., Arpaia, S., Christiaens, O., Dietz-Pfeilstetter, A., Jones, H., Mezzetti, B., et al. (2020). RNA-based Biocontrol Compounds: Current Status and Perspectives to Reach the Market. *Pest Manag. Sci.* 76, 841–845. doi:10.1002/PS.5686

Taning, C. N. T., Gui, S., De Schutter, K., Jahani, M., Castellanos, N. L., Christiaens, O., et al. (2021a). A Sequence Complementarity-Based Approach for Evaluating Off-Target Transcript Knockdown in *Bombus terrestris*, Following Ingestion of Pest-specific dsRNA. *J. Pest Sci.* 94, 487–503. doi:10.1007/S10340-020-01273-Z/FIGURES/7

Taning, C. N. T., Mezzetti, B., Kleter, G., Smagghe, G., and Baraldi, E. (2021b). Does RNAi-Based Technology Fit within EU Sustainability Goals? *Trends Biotechnol.* 39, 644–647. doi:10.1016/J.TIBTECH.2020.11.008

Tenllado, F., Martínez-García, B., Vargas, M., and Díaz-Ruíz, J. R. (2003). Crude Extracts of Bacterially Expressed dsRNA Can Be Used to Protect Plants against Virus Infections. *BMC Biotechnol.* 3. doi:10.1186/1472-6750-3-3

Thudi, M., Palakurthi, R., Schnable, J. C., Chitikineni, A., Dreisigacker, S., Mace, E., et al. (2021). Genomic Resources in Plant Breeding for Sustainable Agriculture. *J. Plant Physiol.* 257, 153351. doi:10.1016/J.JPLPH.2020.153351

Tomoyasu, Y., Miller, S. C., Tomita, S., Schoppmeier, M., Grossmann, D., and Bucher, G. (2008). Exploring Systemic RNA Interference in Insects: A Genome-wide Survey for RNAi Genes in Tribolium. *Genome Biol.* 9, 1–22. doi:10.1186/GB-2008-9-1-R10/FIGURES/8

Torney, F., Trewyn, B. G., Lin, V. S. Y., and Wang, K. (2007). Mesoporous Silica Nanoparticles Deliver DNA and Chemicals into Plants. *Nat. Nanotechnol.* 25 (2), 295–300. doi:10.1038/nnano.2007.108

Tsai, S. Q., Nguyen, N. T., Malagon-Lopez, J., Topkar, V. V., Aryee, M. J., and Joung, J. K. (2017). CIRCLE-seq: a Highly Sensitive *In Vitro* Screen for Genome-wide CRISPR–Cas9 Nuclease Off-Targets. *Nat. Methods* 146 14, 607–614. doi:10.1038/nmeth.4278

Tsai, S. Q., Zheng, Z., Nguyen, N. T., Liebers, M., Topkar, V. V., Thapar, V., et al. (2014). GUIDE-seq Enables Genome-wide Profiling of Off-Target Cleavage by CRISPR-Cas Nucleases. *Nat. Biotechnol.* 332 33, 187–197. doi:10.1038/nbt.3117

Urnov, F. D., and Wolffe, A. P. (2001). Above and within the Genome: Epigenetics Past and Present. *J. Mammary Gland. Biol. Neoplasia* 6, 153–167. doi:10.1023/A:1011304606604

Uusi-Mäkelä, M. I. E., Barker, H. R., Bäuerlein, C. A., Häkkinen, T., Nykter, M., and Rämet, M. (2018). Chromatin Accessibility Is Associated with CRISPR-Cas9 Efficiency in the Zebrafish (*Danio rerio*). *PLoS One* 13. doi:10.1371/JOURNAL.PONE.0196238

van Butselaar, T., and Van den Ackerveken, G. (2020). Salicylic Acid Steers the Growth–Immunity Tradeoff. *Trends Plant Sci.* 25, 566–576. doi:10.1016/J.TPLANTS.2020.02.002

Veillet, F., Perrot, L., Guyon-Debast, A., Kermarrec, M. P., Chauvin, L., Chauvin, J. E., et al. (2020). Expanding the CRISPR Toolbox in P. patens Using SpCas9-NG Variant and Application for Gene and Base Editing in Solanaceae Crops. *Int. J. Mol. Sci.* 21 (102421), 1024. doi:10.3390/IJMS21031024

Vergani-Junior, C. A., Tonon-da-Silva, G., Inan, M. D., and Mori, M. A. (2021). DICER: Structure, Function, and Regulation. *Biophys. Rev.* 13, 1081–1090. doi:10.1007/S12551-021-00902-W

Verkuijl, S. A., and Rots, M. G. (2019). The Influence of Eukaryotic Chromatin State on CRISPR-Cas9 Editing Efficiencies. *Curr. Opin. Biotechnol.* 55, 68–73. doi:10.1016/J.COPBIO.2018.07.005

Wada, N., Ueta, R., Osakabe, Y., and Osakabe, K. (2020). Precision Genome Editing in Plants: State-Of-The-Art in CRISPR/Cas9-based Genome Engineering. *BMC Plant Biol.* 20, 1–12. doi:10.1186/S12870-020-02385-5/TABLES/2

Waltz, E. (2016). Gene-edited CRISPR Mushroom Escapes US Regulation. *Nature* 532, 293. doi:10.1038/nature.2016.19754

Wang, E., and Hunter, C. P. (2017). SID-1 Functions in Multiple Roles to Support Parental RNAi in caenorhabditis Elegans. *Genetics* 207, 547–557. doi:10.1534/GENETICS.117.300067/-/DC1

Wang, H., Gong, L., Qi, J., Hu, M., and Zhong, G. (2014a). Molecular Cloning and Characterization of a SID-1-like Gene in Plutella xylostella. *Arch. Insect Biochem. Physiol.* 87, 164–176. doi:10.1002/ARCH.21189

Wang, K., Peng, Y., Chen, J., Peng, Y., Wang, X., Shen, Z., et al. (2020a). Comparison of Efficacy of RNAi Mediated by Various Nanoparticles in the Rice Striped Stem Borer (Chilo Suppressalis). *Pestic. Biochem. Physiol.* 165, 104467. doi:10.1016/J.PESTBP.2019.10.005

Wang, M., Weiberg, A., Lin, F. M., Thomma, B. P. H. J., Huang, H. Da, and Jin, H. (2016). Bidirectional Cross-Kingdom RNAi and Fungal Uptake of External RNAs Confer Plant Protection. *Nat. Plants* 210 (2), 1–10. doi:10.1038/nplants.2016.151

Wang, M., Xu, Z., Gosavi, G., Ren, B., Cao, Y., Kuang, Y., et al. (2020b). Targeted Base Editing in Rice with CRISPR/ScCas9 System. *Plant Biotechnol. J.* 18, 1645. doi:10.1111/PBI.13330

Wang, Y., Cheng, X., Shan, Q., Zhang, Y., Liu, J., Gao, C., et al. (2014b2014). Simultaneous Editing of Three Homoeoalleles in Hexaploid Bread Wheat Confers Heritable Resistance to Powdery Mildew. *Nat. Biotechnol.* 329 32, 947–951. doi:10.1038/nbt.2969

Weiberg, A., Wang, M., Lin, F. M., Zhao, H., Zhang, Z., Kaloshian, I., et al. (2013). Fungal Small RNAs Suppress Plant Immunity by Hijacking Host RNA Interference Pathways. *Science* 342, 118–123. doi:10.1126/SCIENCE.1239705

Whangbo, J. S., Weisman, A. S., Chae, J., and Hunter, C. P. (2017). SID-1 Domains Important for dsRNA Import in *Caenorhabditis elegans*. *G3 Genes., Genomes, Genet.* 7, 3887–3899. doi:10.1534/G3.117.300308/-/DC1/FIGURES1.PDF

Wienert, B., Wyman, S. K., Richardson, C. D., Yeh, C. D., Akcakaya, P., Porritt, M. J., et al. (2019). Unbiased Detection of CRISPR Off-Targets *In Vivo* Using DISCOVER-Seq. *Science* 364, 286. doi:10.1126/SCIENCE.AAV9023

Wilson, R. C., and Doudna, J. A. (2013). Molecular Mechanisms of RNA Interference. *Annu. Rev. Biophys.* 42, 217–239. doi:10.1146/ANNUREV-BIOPHYS-083012-130404

Winston, W. M., Molodowitch, C., and Hunter, C. P. (2002). Systemic RNAi in *C. elegans* Requires the Putative Transmembrane Protein SID-1. *Science* 295, 2456–2459. doi:10.1126/SCIENCE.1068836

Wu, Y., He, Y., Sretenovic, S., Liu, S., Cheng, Y., Han, Y., et al. (2022). CRISPR-BETS: a Base-Editing Design Tool for Generating Stop Codons. *Plant Biotechnol. J.* 20, 499–510. doi:10.1111/PBI.13732

Wynant, N., Santos, D., Van Wielendaele, P., and Vanden Broeck, J. (2014). Scavenger Receptor-Mediated Endocytosis Facilitates RNA Interference in the Desert Locust, *Schistocerca gregaria*. *Insect Mol. Biol.* 23, 320–329. doi:10.1111/IMB.12083

Wytinck, N., Sullivan, D. S., Biggar, K. T., Crisostomo, L., Pelka, P., Belmonte, M. F., et al. (2020). Clathrin Mediated Endocytosis Is Involved in the Uptake of Exogenous Double-Stranded RNA in the White Mold Phytopathogen Sclerotinia sclerotiorum. *Sci. Rep.* 10, 1–12. doi:10.1038/s41598-020-69771-9

Xie, X., Ma, X., Zhu, Q., Zeng, D., Li, G., and Liu, Y. G. (2017). CRISPR-GE: A Convenient Software Toolkit for CRISPR-Based Genome Editing. *Mol. Plant* 10, 1246–1249. doi:10.1016/J.MOLP.2017.06.004

Xing, S., Chen, K., Zhu, H., Zhang, R., Zhang, H., Li, B., et al. (2020). Fine-tuning Sugar Content in Strawberry. *Genome Biol.* 21, 1–14. doi:10.1186/S13059-020-02146-5/FIGURES/5

Xiong, J. S., Ding, J., and Li, Y. (2015). Genome-editing Technologies and Their Potential Application in Horticultural Crop Breeding. *Hortic. Res.* 21 2, 1–10. doi:10.1038/hortres.2015.19

Xu, W., and Han, Z. (2008). Cloning and Phylogenetic Analysis of Sid-1-like Genes from Aphids. *J. Insect Sci.* 8. doi:10.1673/031.008.3001

Xu, W., Song, W., Yang, Y., Wu, Y., Lv, X., Yuan, S., et al. (2019). Multiplex Nucleotide Editing by High-Fidelity Cas9 Variants with Improved Efficiency in Rice. *BMC Plant Biol.* 19, 1–10. doi:10.1186/S12870-019-2131-1/FIGURES/3

Yan, S., Ren, B. Y., and Shen, J. (2021). Nanoparticle-mediated Double-Stranded RNA Delivery System: A Promising Approach for Sustainable Pest Management. *Insect Sci.* 28, 21–34. doi:10.1111/1744-7917.12822

Yin, K., Han, T., Liu, G., Chen, T., Wang, Y., Yu, A. Y. L., et al. (2015). A Geminivirus-Based Guide RNA Delivery System for CRISPR/Cas9 Mediated Plant Genome Editing. *Sci. Rep.* 51 (5), 1–10. doi:10.1038/srep14926

Yong, W. S., Hsu, F. M., and Chen, P. Y. (2016). Profiling Genome-wide DNA Methylation. *Epigenetics Chromatin* 91 9, 1–16. doi:10.1186/S13072-016-0075-3

Yoon, J. S., Gurusamy, D., and Palli, S. R. (2017). Accumulation of dsRNA in Endosomes Contributes to Inefficient RNA Interference in the Fall Armyworm, Spodoptera Frugiperda. *Insect biochem. Mol. Biol.* 90, 53–60. doi:10.1016/J.IBMB.2017.09.011

Young, J., Zastrow-Hayes, G., Deschamps, S., Svitashev, S., Zaremba, M., Acharya, A., et al. (2019). CRISPR-Cas9 Editing in Maize: Systematic Evaluation of Off-Target Activity and its Relevance in Crop Improvement. *Sci. Rep.* 91 9, 1–11. doi:10.1038/s41598-019-43141-6

Younis, A., Siddique, M. I., Kim, C. K., and Lim, K. B. (2014). RNA Interference (RNAi) Induced Gene Silencing: A Promising Approach of Hi-Tech Plant Breeding. *Int. J. Biol. Sci.* 10, 1150–1158. doi:10.7150/ijbs.10452

Yu, J., Tu, L., Subburaj, S., Bae, S., and Lee, G. J. (2021). Simultaneous Targeting of Duplicated Genes in Petunia Protoplasts for Flower Color Modification via CRISPR-Cas9 Ribonucleoproteins. *Plant Cell. Rep.* 40, 1037–1045. doi:10.1007/S00299-020-02593-1/FIGURES/3

Yu, Q. H., Wang, B., Li, N., Tang, Y., Yang, S., Yang, T., et al. (2017). CRISPR/Cas9-induced Targeted Mutagenesis and Gene Replacement to Generate Long-Shelf Life Tomato Lines. *Sci. Rep.* 71 7, 1–9. doi:10.1038/s41598-017-12262-1

Zetsche, B., Gootenberg, J. S., Abudayyeh, O. O., Slaymaker, I. M., Makarova, K. S., Essletzbichler, P., et al. (2015). Cpf1 Is a Single RNA-Guided Endonuclease of a Class 2 CRISPR-Cas System. *Cell.* 163, 759–771. doi:10.1016/J.CELL.2015.09.038/ATTACHMENT/D7072A0B-0438-4D32-89BF-8FCD238CBAEA/MMC3.XLSX

Zhang, L., Hou, D., Chen, X., Li, D., Zhu, L., Zhang, Y., et al. (2011). Exogenous Plant MIR168a Specifically Targets Mammalian LDLRAP1: Evidence of Cross-Kingdom Regulation by microRNA. *Cell. Res.* 221 22, 107–126. doi:10.1038/cr.2011.158

Zhang, T., Zhao, Y. L., Zhao, J. H., Wang, S., Jin, Y., Chen, Z. Q., et al. (2016). Cotton Plants Export microRNAs to Inhibit Virulence Gene Expression in a Fungal Pathogen. *Nat. plants* 2. doi:10.1038/NPLANTS.2016.153

Zhang, X. H., Tee, L. Y., Wang, X. G., Huang, Q. S., and Yang, S. H. (2015). Off-target Effects in CRISPR/Cas9-mediated Genome Engineering. *Mol. Ther. - Nucleic Acids* 4, e264. doi:10.1038/MTNA.2015.37

Zhang, Y., Iaffaldano, B., and Qi, Y. (2021a). CRISPR Ribonucleoprotein-Mediated Genetic Engineering in Plants. *Plant Commun.* 2, 100168. doi:10.1016/J.XPLC.2021.100168

Zhang, Y., Massel, K., Godwin, I. D., and Gao, C. (2018). Applications and Potential of Genome Editing in Crop Improvement 06 Biological Sciences 0604 Genetics 06 Biological Sciences 0607 Plant Biology 07 Agricultural and Veterinary Sciences 0703 Crop and Pasture Production. *Genome Biol.* 19, 1–11. doi:10.1186/S13059-018-1586-Y/FIGURES/2

Zhang, Y., Pribil, M., Palmgren, M., and Gao, C. (2020). A CRISPR Way for Accelerating Improvement of Food Crops. *Nat. Food* 14 (1), 200–205. doi:10.1038/s43016-020-0051-8

Zhang, Y., Ren, Q., Tang, X., Liu, S., Malzahn, A. A., Zhou, J., et al. (2021b). Expanding the Scope of Plant Genome Engineering with Cas12a Orthologs and Highly Multiplexable Editing Systems. *Nat. Commun.* 121 12, 1–11. doi:10.1038/s41467-021-22330-w

Zhao, J. H., Zhang, T., Liu, Q. Y., and Guo, H. S. (2021). Trans-kingdom RNAs and Their Fates in Recipient Cells: Advances, Utilization, and Perspectives. *Plant Commun.* 2, 100167. doi:10.1016/J.XPLC.2021.100167

Zheng, Y., Hu, Y., Yan, S., Zhou, H., Song, D., Yin, M., et al. (2019). A Polymer/detergent Formulation Improves dsRNA Penetration through the Body Wall and RNAi-Induced Mortality in the Soybean Aphid Aphis Glycines. *Pest Manag. Sci.* 75, 1993–1999. doi:10.1002/PS.5313

Zhi, L., Su, X., Yin, M., Zhang, Z., Lu, H., Niu, Z., et al. (2021). Genetical Engineering for NK and T Cell Immunotherapy with CRISPR/Cas9 Technology: Implications and Challenges. *Cell. Immunol.* 369. doi:10.1016/J.CELLIMM.2021.104436

Zhong, Z., Sretenovic, S., Ren, Q., Yang, L., Bao, Y., Qi, C., et al. (2019). Improving Plant Genome Editing with High-Fidelity xCas9 and Non-canonical PAM-Targeting Cas9-NG. *Mol. Plant* 12, 1027–1036. doi:10.1016/J.MOLP.2019.03.011

Zhu, C., Bortesi, L., Baysal, C., Twyman, R. M., Fischer, R., Capell, T., et al. (2017). Characteristics of Genome Editing Mutations in Cereal Crops. *Trends Plant Sci.* 22, 38–52. doi:10.1016/J.TPLANTS.2016.08.009

Zhu, H., Li, C., and Gao, C. (2020). Applications of CRISPR–Cas in Agriculture and Plant Biotechnology. *Nat. Rev. Mol. Cell. Biol.* 2111 21, 661–677. doi:10.1038/s41580-020-00288-9

Zischewski, J., Fischer, R., and Bortesi, L. (2017). Detection of On-Target and Off-Target Mutations Generated by CRISPR/Cas9 and Other Sequence-specific Nucleases. *Biotechnol. Adv.* 35, 95–104. doi:10.1016/j.biotechadv.2016.12.003

Zotti, M., dos Santos, E. A., Cagliari, D., Christiaens, O., Taning, C. N. T., and Smagghe, G. (2018). RNA Interference Technology in Crop Protection against Arthropod Pests, Pathogens and Nematodes. *Pest Manag. Sci.* 74, 1239–1250. doi:10.1002/PS.4813

Conflict of Interest: TC and HC were employed by the SEMPRE AgTech.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Bioengineering and Biotechnology

TYPE Opinion
PUBLISHED 19 August 2022
DOI 10.3389/fbioe.2022.912793

OPEN ACCESS

EDITED BY
Karen Hokanson,
University of Minnesota Twin Cities,
United States

REVIEWED BY
Anna Whitfield,
North Carolina State University,
United States

*CORRESPONDENCE
Patricia Machado Bueno Fernandes,
patricia.fernandes@ufes.br

SPECIALTY SECTION
This article was submitted to Biosafety and Biosecurity,
a section of the journal
Frontiers in Bioengineering and Biotechnology

RECEIVED 04 April 2022
ACCEPTED 27 July 2022
PUBLISHED 19 August 2022

CITATION
Fernandes PMB, Favaratto L, Fernandes AAR, Vicien C, Capalbo DMF and Zerbini FM (2022), To become more sustainable organic agriculture needs genome editing technology.
Front. Bioeng. Biotechnol. 10:912793.
doi: 10.3389/fbioe.2022.912793

To become more sustainable organic agriculture needs genome editing technology

Patricia Machado Bueno Fernandes[1]*, Luíza Favaratto[1], A. Alberto R. Fernandes[1], Carmen Vicien[2], Deise M F Capalbo[3] and Francisco Murilo Zerbini[4]

[1]Biotechnology Core, Federal University of Espírito Santo, Vitória, Brazil, [2]Faculty of Agronomy, University of Buenos Aires and Institute for Scientific Cooperation in Environment and Health, Buenos Aires, Argentina, [3]R&D Department, Embrapa Environment, Jaguariúna, Brazil, [4]Department of Plant Pathology, Federal University of Viçosa, Viçosa, Brazil

KEYWORDS

global warming1, CRISPR2, food insecurity3, food regulation4, market release5

1 Introduction

Worldwide, the area identified as "organic agriculture" comprises ca. 72.3 million hectares, with an average yearly growth of 10%. In 2019 the global market of organic foods and drinks reached more than 106 billion euros (FAO 2021). With this area and growth, organic agriculture is already an important player in global food production. Nevertheless, the positive environmental effects of organic farming are less evident when considering food production in kilograms rather than per hectare of cultivated land, mostly because of lower crop yields due to several factors. This leads to the necessity of more land in the case of organic farming, compared to the traditional way, to obtain a similar amount of food as an output (Willer et al., 2021).

In general, regulations of organic production exist under the umbrella of a larger framework of public policies aimed at the adoption of sustainable agricultural practices and the conservation of agroecosystems, focused on food and nutritional security of the population, fairer trade relations, and conscious consumption. Agriculture is heavily affected by the climate crisis, while also representing one of the major sources of greenhouse gas emissions (UNF 2021). The internationally recognized greenhouse gasses covered under the United Nations Framework Convention on Climate Change include carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O), and carbon monoxide (CO). The Gas Emission Estimation System (SEEG 2022) shows that agriculture has a prominent role in the emissions of those greenhouse gasses, especially CH_4 and N_2O.

The world population is predicted to reach 9.7 billion in 2050 (United Nations 2019). According to the World Hunger Clock, in March 2022, approximately 2.4 billion people live in moderate and severe food insecurity. That food production must increase in order to fight this foreseen insecurity is self-evident, but this needs to be done while also ensuring the achievement of the Sustainable Development Goals (SDG). Incorporating new technologies is one major way of reaching this objective and helping to solve the climate crisis.

2 Relationship between organic agriculture and biotechnology

Historically, the relationship between organic agriculture and biotechnology has been antagonistic (Husaini and Sohail 2018). Indeed, a true ideological war has been pursued for years between supporters of organic versus biotechnological agriculture. This antagonism induced many smallholder farmers to believe that there is a complete incompatibility between the two agricultural systems (Purnhagen et al., 2021). This struggle resulted in a legal framework for organic farming which prevents farmers from incorporating GMOs into their production systems, even if it would allow for better quality, increased climate-related resilience, and productivity, and even less use of pesticides. As a result, organic farmers view biotechnology as unnatural and opposed to the principles that drive organic agriculture (IFOAM 2016).

Biotechnology is thus associated with industrial, commodity-based farming, monoculture, intensive use of pesticides, and patented seeds. One of the biggest misconceptions of the organic foundation is to confuse biotechnology - a production process - with an intrinsically unsafe and hazardous product. This misconception is in large part the result of the extreme regulatory framework to which biotech crops are subjected in most countries. In Brazil, for example, obtaining a permit for the "planned release" of most GM plants requires (among other things) detailed information on the dissemination of GM pollen into the environment, on all plant species with which the GM species could possibly cross, and the long-term effects of such crosses. Requirements for a commercial release are orders of magnitude more complex. This difficulty seems to be a constant in most countries. In the European Community, China, and Japan, important players in this subject, there are even more restrictive requirements. It is essentially impossible for an overworked researcher in an understaffed public university or research institute to satisfy all these requirements. Thus, only the large agribusiness companies, with fully staffed compliance departments and plentiful resources, are capable of obtaining such permits. The unfortunate outcome of this ideological war is an aversion and prohibition of GM crops which in reality could be extremely helpful and are completely compatible with organic, sustainable agriculture, and which have no detectable differences regarding food or environmental safety.

3 CRISPRized plants to organic farming

A new window of opportunities for organic agriculture presents itself with the advent of gene-editing technologies such as CRISPR-Cas9. Clustered, regularly interspaced short palindromic repeats (CRISPR) - associated proteins is a technology for genome editing that enables the knock-in and/or knock-out of target genes in specific genome regions (Doudna and Charpentier 2014). This strategy has been successfully applied in model plants, such as Arabidopsis and tobacco, and in crops, as presented in Figure 1, to modify endogenous protein-coding genes (GLP 2020).

It is known that mutagenesis may occur naturally or through long processes of genetic selection. The CRISPR-Cas9 technique made gene editing possible with the purpose of inducing important properties in plant development without necessarily introducing an exogenous gene (Waltz 2016a, 2016b; Nishitani et al., 2016; Shi et al., 2017; Waltz 2018; Jansing et al., 2019; Lyzenga et al., 2019; Gramazio et al., 2020; Cai et al., 2022). In this way, this biotechnological tool eliminates one of the major points raised against biotech crops, which is the "unnatural" insertion of an exogenous gene into the plant's genome. It is imperative to note that, to date, no commercial platform exists enabling the detection of CRISPR-Cas-induced genome edits. Thus, genome editing through CRISPR-Cas is a way of accelerating the production of improved cultivars in a completely safe and sustainable fashion. As national and supranational regulators (such as the Brazilian CTNBio and CONABIA in Argentina, and the European Commission, respectively) engage in debates on whether (and how) to regulate crops obtained with the use of CRISPR-Cas-based and other genome-editing technologies, it is imperative that the nature of genome editing be understood, to avoid the same mistakes made when regulating GM crops, of introducing excessive (and unnecessary) regulations which prevent the widespread use of the technology beyond a few major commodities. To deny the benefits of this revolutionary technology to organic and smallholder farmers would be a tragedy of immense proportions.

4 The way forward–can biotechnology and organic agriculture become partners instead of enemies?

Forty years after the first GM product came on the market (human insulin produced in bacteria; Itakura et al., 1977), the discussion about the safety of GMOs still reverberates. In the 1980's, the first transgenic tobacco, maize, and wheat plants appeared in the United States, and in 1994, the first GM food (the Flavr Savr™ tomato) arrived in American supermarkets (Kramer and Redenbaugh 1994). 30 years later, despite growing scientific evidence that GMOs are as safe as conventional crops–and in fact can bring important benefits for food security and the environment–they remain rejected by organic regulations. This situation represents a true predicament for the advancement of organic farming (Husaini and Sohail 2018).

To cite one of the many statements around the safety of products from modern biotechnology and their potential to help in SDG and overcome environmental problems, a recent study in Spain (Vega Rodríguez et al., 2022) showed that GMOs can serve as nutraceuticals and edible vaccines without the need for broad-

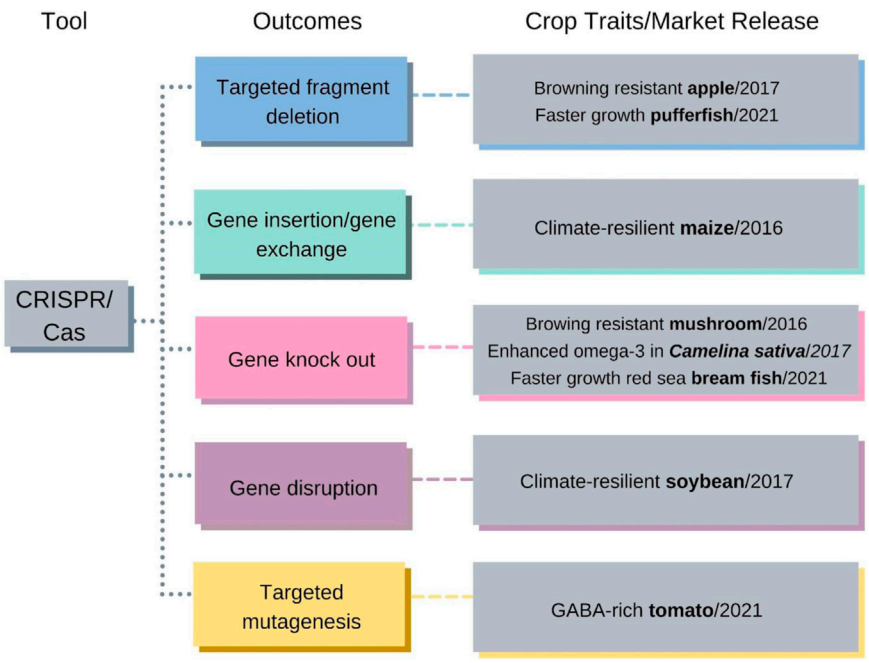


FIGURE 1
CRISPR/Cas and its outcomes and characteristics of crops already available on the market. Colored arrows and boxes link published crop trait examples and year of market release with the associated genome-editing tool and outcome (Jansing et al., 2019). Copyright 2019, MDPI, Basel, Switzerland.

scale industrial facilities for production. Thus, genetically edited foods need to be treated as traditional foods, and food security needs to be prioritized over the methods by which genetic modification/edition traits and properties were incorporated. The researchers also emphasized that debates over modern foods should be based on scientific evidence rather than emotions. Consumer health benefits need to be made known to the public to dispel skepticism related to biotechnology.

There is an urgent need to provide mechanisms so that scientific and technological knowledge is available to all, including the organic farmers and consumers who could benefit significantly from the application of the newest genome-editing technologies to crop improvement. If biotechnology and organic agriculture become partners, both will benefit. But the ultimate winner will be the general population, who will have access to food products that are nutritional, safe, and produced in a sustainable fashion.

CRISPR technology provides the perfect opportunity for this partnership to happen. It is easy to implement, affordable, and, if regulatory hurdles are not unfeasible, its derived seeds will be viable for small family farmers, the basis of organic agriculture. The CRISPR genome editing technology is not only equivalent to traditional breeding technique but actually much more controlled and faster. It should be embraced by the adepts of organic agriculture. We believe that the long-overdue partnership between biotechnology and organic agriculture is fundamental for the mitigation of food insecurity and is the only way forward to a truly sustainable agriculture (World Hunger Clock, 2021).

Author contributions

All authors contributed to the manuscript and approved the final version.

Funding

This manuscript was financially supported on its publication fees by the International Life Science Institute–ILSI–Brazil–Biotec Task Force.

Acknowledgments

PF and FZ acknowledge the Conselho Nacional de Desenvolvimento Científico e Tecnológico–CNPq for their research productivity award (grants # 308306/2021-0 and 312493/2018-5, respectively). LF acknowledges Fundação de Apoio à Pesquisa e Inovação do Espírito Santo–FAPES for her

scholarship. The authors thanks A C Gianotto for fruitful discussion.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

Cai, Z-X., Chen, M-Y., Lu, Y-P., Guo, Z-J., Zeng, Z-H., Liao, J-H., et al. (2022). Metabolomics and transcriptomics unravel the mechanism of browning resistance in agaricus bisporus. *Plos One* 17 (3), e0255765. doi:10.1371/journal.pone.0255765

Doudna, J. A., and Charpentier, E. (2014). The new frontier of genome engineering with CRISPR-Cas9. *Science* 346 (6213), 1258096. doi:10.1126/science.1258096

FAO (2021). *The world of organic agriculture*. Available at: https://www.fao.org/family-farming/detail/en/c/1378841/Accessed March 11, 2022).

GLP (2020). *United States: Crops/Food*. Available at: https://crispr-gene-editing-regs-tracker.geneticliteracyproject.org/united-states-crops-food/Accessed March 13, 2022).

Gramazio, P., Takayama, M., and Ezura, H. (2020). Challenges and prospects of new plant breeding techniques for GABA improvement in crops: Tomato as an example. *Front. Plant Sci.* 11, 1–16. doi:10.3389/fpls.2020.577980

Husaini, A. M., and Sohail, M. (2018). Time to redefine organic agriculture: Can't GM crops Be certified as organics? *Front. Plant Sci.* 9, 423. doi:10.3389/fpls.2018.00423

IFOAM (2016). *Organics international position on genetic engineering and genetically modified organisms*. Brussels: Draft for Public Consultation. Available at: http://www.ifoam.bio/en/news/2016/02/26/public-consultation-position-ifoam-organics-international-genetic-engineering-and Accessed March 23, 2022).

Itakura, K., Hirose, T., Crea, R., Riggs, A. D., Heyneker, H. L., Bolivar, F., et al. (1977). Expression in Escherichia coli of a chemically synthesized gene for the hormone somatostatin. *Science* 198 (4321), 1056–1063. doi:10.1126/science.412251

Jansing, J., Schiermeyer, A., Schillberg, S., Fischer, R., and Bortesi, L. (2019). Genome editing in agriculture: Technical and practical considerations. *Int. J. Mol. Sci.* 20 (12), 2888. doi:10.3390/ijms20122888

Kramer, M. G., and Redenbaugh, K. (1994). Commercialization of a tomato with an antisense polygalacturonase gene: The FLAVR SAVR tomato story. *Euphytica* 79 (3), 293–297. doi:10.1007/BF00022530

Lyzenga, W. J., Harrington, M., Bekkaoui, D., Wigness, M., Hegedus, D. D., and Rozwadowski, K. L. (2019). CRISPR/Cas9 editing of three CRUCIFERIN C homoeologues alters the seed protein profile in camelina sativa. *BMC Plant Biol.* 19 (1), 1–16. doi:10.1186/s12870-019-1873-0

Nishitani, C., Hirai, N., Komori, S., Wada, M., Okada, K., Osakabe, K., et al. (2016). Efficient genome editing in apple using a CRISPR/Cas9 system. *Sci. Rep.* 6, 1–8. doi:10.1038/srep31481

Purnhagen, K. P., Clemens, S., Eriksson, D., Fresco, L. O., Tosun, J., Qaim, M., et al. (2021). Europe's farm to fork strategy and its commitment to biotechnology and organic farming: Conflicting or complementary Goals? *Trends Plant Sci.* 26, 600–606. doi:10.1016/j.tplants.2021.03.012

SEEG (2022). *Total gasses emission*. Available at: https://plataforma.seeg.eco.br/total_emission Accessed March 22, 2022).

Shi, J., Gao, H., Wang, H., Lafitte, H. R., Archibald, R. L., Yang, M., et al. (2017). ARGOS8 variants generated by CRISPR-cas9 improve maize grain yield under field drought stress conditions. *Plant Biotechnol. J.* 15 (2), 207–216. doi:10.1111/pbi.12603

UNF (2021). *Climate issues to watch in 2022: A year for more action and bigger ambition*. Available at: https://unfoundation.org/blog/post/climate-issues-to-watch-in-2022-a-year-for-more-action-and-bigger-ambition/?gclid=CjwKCAjwlcaRBhBYEiwAK341jcjn-HEr8YqUsd721pNjk5zJUZup-Y-JmwNUVWX1yX2FsK0f4yZ7-BoCYN0QAvD_BwE Accessed March 19, 2022).

United Nations (2019). *Growing at a slower pace, world population is expected to reach 9.7 billion in 2050 and could peak at nearly 11 billion around 2100*. Available at: https://www.un.org/development/desa/en/news/population/world-population-prospects-2019.html Accessed March 20, 2022).

Vega Rodríguez, A., Rodríguez-Oramas, C., Sanjuán Velázquez, E., Hardisson de la Torre, A., Rubio Armendáriz, C. R., and Carrascosa Iruzubieta, C. C. (2022). Myths and realities about genetically modified food: A risk-benefit analysis. *Appl. Sci.* 12, 2861. doi:10.3390/app12062861

Waltz, E. (2016a). CRISPR-edited crops free to enter market, skip regulation. *Nat. Biotechnol.* 34 (6), 582. doi:10.1038/nbt0616-582

Waltz, E. (2016b). Gene-edited CRISPR mushroom escapes US regulation. *Nature* 532 (7599), 293. doi:10.1038/nature.2016.19754

Waltz, E. (2018). With a free pass, CRISPR-edited plants reach market in record time. *Nat. Biotechnol.* 36 (1), 6–7. doi:10.1038/nbt0118-6b

Willer, H., Trávníček, J., Meier, C., and Schlatter, B. (2021). *The world of organic agriculture statistics and emerging trends 2021*. Available at: https://shop.fibl.org/de/artikel/c/statistik/p/1663-organic-world-2015.html.

World Hunger Clock (2021). *World hunger Clock*. Available at: https://worldhunger.io/?campaignid=11650289004&adgroupid=114186627555&adid=484276842476&gclid=CjwKCAjwlcaRBhBYEiwAK341jaRVrDlkUvH3xEQv1wpSB5QJSRGMIpguul_7kEW-XHj96o_hJ463hBoCD60QAvD_BwE Accessed March 15, 2022).

frontiers | Frontiers in Bioengineering and Biotechnology

TYPE Original Research
PUBLISHED 30 August 2022
DOI 10.3389/fbioe.2022.977793

OPEN ACCESS

EDITED BY
Carmen Vicien,
University of Buenos Aires, Argentina

REVIEWED BY
Phetole Mangena,
University of Limpopo, South Africa
Stuart Smyth,
University of Saskatchewan, Canada

*CORRESPONDENCE
José Maria Ferreira Jardim da Silveira,
jmsilv52@gmail.com

SPECIALTY SECTION
This article was submitted to Biosafety and Biosecurity,
a section of the journal
Frontiers in Bioengineering and Biotechnology

RECEIVED 24 June 2022
ACCEPTED 01 August 2022
PUBLISHED 30 August 2022

CITATION
Seixas RNdL, Silveira JMFJd and Ferrari VE (2022), Assessing environmental impact of genetically modified seeds in Brazilian agriculture. *Front. Bioeng. Biotechnol.* 10:977793. doi: 10.3389/fbioe.2022.977793

Assessing environmental impact of genetically modified seeds in Brazilian agriculture

Renato Nunes de Lima Seixas[1],
José Maria Ferreira Jardim da Silveira[2]* and
Vinícius Eduardo Ferrari[3]

[1]Economics Department, Federal University of Espírito Santo, Vitória, Espírito Santo, Brazil, [2]Institute of Economics, State University of Campinas, Campinas, São Paulo, Brazil, [3]Post-Graduation Program in Sustainability, Center for Economics and Administration, Pontifical Catholic University of Campinas, Campinas, São Paulo, Brazil

Genetically modified (GM) seeds have had relevant impacts on worldwide agriculture, even with a limited number of essential traits launched in the markets. The focus on platforms crops has favored the combination of traditional breeding, GM insertion, and diffusion in agriculture. One of the remarkable features of the GM traits has been the close link with pest and weed control systems. We investigate the environmental effects due to pesticides for two different GM seeds: insect resistant (IR) cotton and herbicide tolerant (HT) soybeans in a particular period of Brazilian agriculture, 2009–2013. We use a dataset on commercial farms' use of pesticides and biotechnology in Brazil to document environmental effects of GM traits. We explore within farm variation for farmers planting conventional and GM seeds to identify the effect of adoption on the environmental impact of pesticides measured as the quantity of active ingredients of chemicals and the Environmental Impact Quotient (EIQ) index. The findings show that the IR trait reduces application of insecticides by 22% and the associated environmental impact by 20% the environmental impact of insecticides. However, for HT traits, we find that application of herbicides increases by 55.8% and the associated environmental impact by 44.4%, showing a significant increase in the EIQ. The HT results are driven by an increase of less toxic herbicides elevenfold larger than the decrease in less toxic ones, which we interpret as evidence of weak substitutability between herbicides of different toxicity levels. Addressing what happened in the last decade, the paper also presents a view of the transformations in GM usage in Brazil, focusing on the considerable success in adopting stacked genes. Future perspectives point to a more diversified menu of technologies, crops, and adopting countries, going beyond platform crops and more prominent agriculture exporters.

KEYWORDS

environmental impact, transgenic seeds, stacked genes, pesticides, new breeding technologies, CRISPR

1 Introduction

GM seeds have been considered one of the major technological innovations for agricultural systems and have been promoted as an effective tool for controlling agricultural pests and expanding food supply. Their relevance can also be measured by the wide span of controversial issues that have been raised in the related literature since their introduction. Those involve intellectual property rights over organisms, productivity effects, economic returns, consumer safety, welfare and income distribution, and environmental effects (Graff et al., 2003; Qaim, 2009; Carpenter, 2010; Barrows et al., 2014; Maia and Silveira, 2016; Ferrari et al., 2021). Potential sources of related economic gains include reduced crop losses, reduced expenditure on pest control, farmworker safety and health conditions, lower variability of output and consequently, less risk (Sexton and Zilberman, 2012; Smyth et al., 2015; Krishna et al., 2016; Alves et al., 2020). There is also a concern with the non-GM markets regarding the lack of availability of inputs and price differentials (Kalaitzandonakes et al., 2018; Punt and Wesseler, 2018; Oliveira et al., 2020).

Since the mid 1990s, when first-generation GM seeds were commercially introduced, adoption by farmers has grown steadily in industrialized and developing countries as they provide an alternative and more convenient way of controlling weeds and pest damage. By 2018, farmers of 26 countries have cultivated 199.5 million hectares to GM seeds, about 90% of them corresponding to small farmers (ISAAA, 2018). From the first approval of a GM seed in 1996–2018, the number of hectares cultivated with GM grew persistently at 12.8% per year. The main reasons are: 1) the successive approval of GM platform crops (soybean, corn, cotton, and canola) of IR, HT, and IR + HT events in the leading grain producers in the world, notably the US, Canada, Argentina, Brazil, Paraguay, Uruguay, and in the critical grain consumers, India and China; 2) the approval of new traits, highlighting drought resistance sugarcane in Indonesia; 3) the expansion to other countries, like Mexico, Vietnam, and Pakistan - data from ISAAA (2018, p. 7).

From 2006 to 2018, the growth rate is shallow, not significantly different from zero. The main reason is the rapid diffusion of the two main events in the big agricultural countries, reaching the top of 90% of adoption, a huge success. The deceleration is not compensated by the emergence of new countries and new events. Only Portugal and Spain have adopted GM crops in Europe, reflecting the persistence of bans (Oliveira et al., 2020). The heterogeneity of the diffusion processes has been firmly determined by the gains from adoption in the leading agricultural exporters in the world in comparison with other agricultural countries, according to Brookes and Barfoot (2018, 2020).

On the environmental front, benefits related to adoption of GM seeds have been argued based on findings about pesticide use and agricultural practices (Klümper and Qaim, 2014; Datta et al., 2019; Kranthi and Stone, 2020). Insect resistant (IR) cotton has been found to reduce the use of insecticides and therefore to produce environmental, health and safety gains (Huang et al., 2002; Qaim and Zilberman, 2003; Qaim and Janvry, 2005; Qiao, 2015; Veettil et al., 2017). Tabashnik and Carrière (2017) analyze the global monitoring data reported during the first 2 decades of transgenic crops and identified the increase of pest resistance to Bt proteins (Cry and Vip)[1]. They suggested adopting agricultural practices to lessen the adverse effects of pest resistance.

Herbicide tolerant (HT) soybeans have been found to change the mix of herbicides applied towards less toxic ones and to allow the use of no-till cultivation techniques, leading researchers to conclude (tentatively) that they also produce environmental benefits (Fernandez-Cornejo et al., 2002; Qaim and Traxler, 2005; Brookes and Barfoot, 2018; Kalaitzandonakes et al., 2018). However, the diffusion of herbicide-tolerant events associated with a minimal variety of herbicides has generated herbicide resistance with a potential of compromising technology value (Smale et al., 2012; Bonny, 2016; Lamichhane et al., 2017; Schütte et al., 2017).

Although the predominantly favorable evaluation of impacts, a report of the US National Academies of Sciences Engineering and Medicine casts doubts on the productivity and environmental gains that were promised when GM seeds were first introduced (National Academies of Sciences, 2016). Based on a thorough review of evidence accumulated over the last two decades, the report concluded that IR traits in cotton and maize crops decreased the gap between potential and actual yields when targeted pests were a significant source of losses even with chemical control. Nevertheless, when examining data on overall yield per hectare for cotton, maize and soybeans reported by the US Department of Agriculture, the report found no evidence that GM traits have substantially increased the rate at which the US is increasing agricultural yields (National Academies of Sciences, 2016).

Regarding pesticides use, the report found that IR traits have decreased the number of insecticide applications and of kilograms of active ingredients per hectare applied on maize and cotton crops. For HT traits, on the other hand, the evidence on the amount of herbicide per hectare of crop is mixed, with studies that found initial decreases in total amount in soybean crops that were not sustained over time, mostly due to increased resistance of weeds to herbicides (National Academies of Sciences, 2016). The report also warns that analysis that find overall increase or decrease in kilograms of herbicides per hectare

1 Insect-resistant genetically modified crops receive genes from *Bacillus thuringiensis*, a Gram-positive bacteria that allows the plant to synthesize proteins that interfere with the absorption of food from insects. The genetic modification uses two types of genes: Cry (parospal crystal) and Vip (vegetative insecticide protein). Combining the different genes of Cry and Vip can amplify the range of insect control by Bt crops.

TABLE 1 Effect of IR trait on quantity and field EIQ of insecticides and total pesticides.

	(1)	(2)	(3)	(4)
	Insecticides	**Total**	**Insecticides**	**Total**
IR trait	−1.025***	−1.005***	−31.449***	−33.237***
	[0.185]	[0.242]	[5.596]	[6.844]
Constant	1.168	3.418**	50.686	111.582**
	[1.146]	[1.131]	[34.214]	[34.097]
N	186	186	186	186
r^2	0.822	0.861	0.848	0.873
Mean of Dep. Var. +	4.67	11.01	154.94	304.66

Models (1) and (2): kg/ha of active ingredients of insecticides and total pesticides.
Models (3) and (4): field EIQ, for insecticides and total pesticides.
Restricted sample: farmers that use conventional and IR, seeds.
Robust standard errors in brackets.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
+Conventional seeds.

can be misleading, since some herbicides are effective at much lower rates than others and changes in applications rates per hectare do not consider changes in the quality mix of herbicides applied.

Recently, some studies raised concerns about the soybean system of weed control, challenging the idea of the social and environmental benefits of the usage of glyphosate in GM crops (Dias et al., 2019). After 25 years following the initial GM diffusion, environmental concerns, and some critics of the performance of GM cultivars, are still in place, even in countries like Brazil and Argentina, which are highly competitive in soybean, corn, and cotton. Brazil is ranked second in GM adoption, justifying the importance of investigating the environmental impacts.

The paper proceeds as follows. The second section evaluates the environmental impact of the use of GM plants that are herbicide tolerant and insect resistant. Field research covers the 2009–2013 period with a particular feature regarding the rapid diffusion of GM varieties: the seed supply was predominantly of non-stacked GM seeds, and many growers use conventional types. One relevant section's contribution is to compare each GM type on the market with the environmental impact of the use of conventional seeds. The section innovates relative to previous works by employing a broader measure of environmental impact that considers toxicity levels and risk of exposure in evaluating the effects of pesticides for different dimensions of the agricultural system. It allows for uncovering environmental impacts that have been hidden by the qualitative nature of the change in the mix of pesticides used.

The third section provides an analysis of the environmental effects related to the use of pesticides arising from the adoption of IR cotton and HT soybean seeds. The fourth section discuss the quick diffusion of stacked GM that partially contributes to reducing the criticism of the environmental impacts, combining the reduction of insecticide usage with the crop management only possible with HT traits to map the new trends in genetic modified crops, from stacked genes in soybean to novelties based on gene editing. The existence of technological variety for soy and mainly in corn confirms the relevance of GM traits to Brazilian agriculture. The second part of the section discusses the future contributions of plant breeding technologies with attention to climate change effects. The fifth section summarizes the contribution of the paper and its main conclusions.

2 Evaluation of environment impact of GM seeds in brazilian agriculture

2.1 Methodology

2.1.1 Formulating the evaluation hypothesis

The environmental impact related to pesticides use of GM seeds in Brazil demands a careful assessment, not presented yet in the literature. In the period 2009 up to 2013, as pointed above, the diffusion of stacked GM seeds was going fast, but it was possible, in this period, to find growers choosing HT or IR, allowing the field research to treat HT and IR traits separately.

Soybean seeds engineered with HT traits are the result of the transfer of part of the genetic code of a soil bacterium, *Agrobacterium tumefaciens*, which allow the plant to metabolize the herbicide glyphosate. In 1998, soybean varieties tolerant to the herbicide glufosinate were introduced under the commercial name Liberty Link. Those herbicides target a large variety of broad-leaf and grass weeds species but cause severe damages to conventional crops when applied after germination (post-emergent weed control). The primary reason given for the rapid diffusion rate of those seeds,

notably the Roundup Ready ones, is the simplicity of the glyphosate-based weed control, which allows farmers to concentrate on one herbicide to control a wide range of weeds. In addition, it also proved more convenient for farmers since the timing of application can be extended beyond soybean flowering and the maximum size of weeds that are effectively controlled is greater compared with other postemergence herbicides (Carpenter & Gianessi, 1999). Herbicide related cost savings have also been pointed as one of the reasons for adoption, since glyphosate patent expired in the year of 2000, allowing the entry of new suppliers, and lowering the price of glyphosate-based herbicides (Qaim, 2009). Hence, from the point of view of farmers, HT soybeans have been shown to be both technically and economically advantageous, which explains the rapid diffusion they have displayed.

This description of the effects of the HT trait on the plant allows us to formulate two working hypotheses on how it changes the amount of herbicides that farmers choose and the corresponding environmental effect. First, since the HT trait makes the plant tolerant to some specific herbicides—the ones with active ingredients that the plant is now able to metabolize—it can be seen as a technical complement to those chemicals. Hence, we expect the HT trait to induce farmers to use more of the herbicides that the plant is tolerant per hectare. As for the environmental effect, since farmers use more of less toxic chemicals, this should be weighted against the way they substitute away from other more toxic herbicides. If this substitution is strong enough, it is possible that the net effect is a reduction on the environmental impact in terms of general toxicity of the weed-control strategy. On the other hand, if this substitution is weak, the net effect would be an increase in the general toxicity of the weed-control strategy since the additional low-toxicity herbicide would be used on top of high-toxicity ones. We summarize these hypotheses as the following:

1) HT trait increases the amount per hectare of some herbicides applied to the crop. Specifically, it increases the amount of herbicides that the plant becomes tolerant to.
2) Since the herbicides that the plant becomes tolerant to are of lower toxicity, the net environmental effect depends on the strength of substitution among herbicides of different toxicity levels.

IR seeds are engineered to produce a natural toxin found in the soil bacterium Bacillus thuringiensis (Bt), which is lethal to a number of caterpillars (rootworms, earworm, bollworms) pests but not to mammals[2]. IR crops have also been considered technically and economically efficient for producers. The most straightforward reason is related to savings in insecticides applications (which spans savings in labor time, machinery use, aerial spraying etc.) targeted to bollworm killing. Specifically, in regions with high insect infestation, typical less developed countries in tropical weather regions, and high rates of insecticide use, the potential for reduction is conversely high (Qaim and Zilberman, 2003; Kathage and Qaim, 2012; ISAAA, 2018).

IR seeds have also been found to increase yields relative to non-GM ones since the toxin produced by the plant, compounded with the insecticides usage, reduces losses due to insect attacks (Qaim, 2009; Veettil et al., 2017). In fact, it has been argued that yield and insecticide reduction effects are closely related: farmers facing high pest pressure and still using low rates of insecticides. Besides, it has also been considered a more efficient tool for managing the risk of pest attack than reactive application of insecticides (Crost and Shankar, 2008) which has been translated in reduced crop insurance premium. Other benefits relate to improved farm workers' safety conditions and shorter growing seasons (Brookes and Barfoot, 2018, 2020).

As for the HT trait, we can formulate two working hypotheses for the effects of the IR trait on the amount of insecticides used and the related environmental impact of the insect-control strategy. Since the plant produces a natural toxin that substitutes insecticides aimed at some types of bollworms, the IR trait works as a substitute for chemical insecticides and hence reduces the amount that farmers have to apply. The environmental effect should be straightforward: fewer chemicals applied to the plant should lead to a less toxic pest control strategy. We summarize these two hypotheses in the following statements:

1) The IR trait reduces the amount of insecticides that farmers apply to the crop.
2) The IR trait reduces the environmental damage related to the application of chemical insecticides.

This discussion suggests that measuring environmental impacts associated with pesticide use is not straightforward. For HT traits, specifically, the net effect on environmental impact is an open issue. Economists that studied it have focused on the change in the mix of herbicides to conclude that there are environmental gains allowed by the use of HT traits. Nevertheless, we argue that weak substitution might undermine this conclusion as we show in the analysis that follows on the next sub-sections.

2.1.2 Empirical strategy

In the empirical analysis, we use a unique farm-level dataset originated from a survey conducted by a Céleres Consultancy, in

2 The paper takes bollworm as the primary reference to the various types of caterpillars causing damage to agriculture. "Bt" technology offers farmers resistance in the plants to major pests such as stem and stalk borers, earworms, cutworms and rootworm (*Spodoptera frugiperda, Diatraea spp, Helicoverpa zea* and *Diabrotica spp*) in maize, bollworm/budworm (*Heliothis sp* and *Helicoverpa*) in cotton, caterpillars (*Helicoverpa armigeru*) in soybeans.

Brazil. The survey collected data on production, revenue, costs, biotechnology adoption and pesticides used. Information on pesticide use was collected for harvest seasons 2009–2013 and covers 1,030 farms.

The dataset is disaggregated by fields, within a farm, cultivated with conventional or GM seeds. In other words, for each farm, we have potentially multiple observations related to fields cultivated with conventional or GM seeds. This setup allows us to explore within-farm variation between fields cultivated with conventional and GM seeds to identify the effect of biotechnology traits on the use of pesticides and corresponding environmental impact. This identification strategy holds constant all farm-level characteristics that might affect simultaneously the choices of pesticide use and biotechnology adoption such as: management skills, input/output prices, location, weather shocks, etc. Hence, for instance, if soybean farmers that adopt biotechnology have some intrinsic preference for pest management strategies that are more intensive in herbicides than mechanical control (like tillage) the effect of GM traits could be overestimated. Likewise, if cotton farmers that adopt IR traits are more efficient and also use less insecticide in their pest management strategies, the effect of IR trait will be underestimated[3]. The use of within farm variation, i.e., comparing the pesticide use and corresponding environmental impact for farmers that cultivate fields with conventional and GM seeds, gets around these sources of bias on the coefficient that measures the effect of the GM trait.

The farms surveyed represent large operations with potentially large environmental impacts associated with the scale of production and pesticides use. For cotton growers, the average total planted area is 1,888.48 ha, ranging from 50 ha to 26,774 ha. For soybean growers, the average total planted area is 857.88 ha ranging from 5 ha to 11,000 ha. In terms of experience, farmers report an average of 26.95 and 33.33 years for cotton and soybeans respectively. This indicates they have a high level of working experience in the activity.

We measure the environment impact as two outcome variables: quantity (Kg/ha) of active ingredients of chemicals and the Environmental Impact Quotient (EIQ) index (Kovach et al., 1992). This measure of environmental impact of pesticides was designed to capture risks associated with both toxicity levels and exposure to chemical pesticides on three components of agricultural systems: farm worker, consumer and ecological. Hence, the EIQ index provides a more complete picture than just the composition of the mix of pesticides used, or the analysis of kilograms of active ingredients applied to crops, allowing for an adequate weighting of pesticides of different toxicity levels (National Academies of Sciences, 2016).

The use of the EIQ index represents a considerable advancement over previous studies that relied on an increased share of less toxic chemicals in the total quantity (Kg/ha) of herbicides applied in HT soybeans fields since this measure cannot capture environmental effects due to substitution between herbicides. Concretely, if the increase in the use of less toxic herbicides is not accompanied by a sufficient decrease in more toxic ones, the new mix of herbicides induced by HT seeds can be more harmful than the one induced by conventional seeds. The EIQ index calculated for field operations allows us to adequately weight pesticides of different toxicity levels and gets around the difficulties of looking only at the quantity mix of pesticides used.

We estimate linear regression models for cotton and soybean crops separately. The dependent variables are quantity (kg/ha) of pesticides used (insecticides for cotton and herbicides for soybean) and EIQ index for each field. The traits considered are the most common ones for each crop: IR for cotton and HT for soybean. The estimated equations have the following form:

$$y_{itf} = \alpha + \beta trait_f + \gamma_i + \theta_t + \varepsilon_{itf}$$

Subscripts i, t and f indicate farmer, year and field (each field cultivated with conventional or GM seed). We include farmers (γ_i) and time dummies (θ_t) that capture farm-specific and year specific effects. Although these variables are orthogonal to the field level effects that we are interested, therefore not affecting the point estimates, they provide efficiency gains in the estimation (lower standard errors) that prove worth keeping them.

2.2 Results

2.2.1 IR traits in cotton

Table 1 shows estimates of the effect of adoption of IR trait in cotton crops for quantities (Kg/ha) of active ingredients of insecticides and total pesticides applied (models 1 and 2) and for the field EIQ for insecticides and total pesticides (models 3 and 4). Estimates are for the sample of farmers that use both conventional and IR seeds[4].

The coefficient of the IR trait indicates that it allows a reduction of 1.025 kg/ha of active ingredients of insecticides applied to cotton fields. For total pesticides the point estimate is a bit lower in magnitude (−1.005) but not statistically different from the coefficient on insecticides. This indicates that reduction in active ingredients comes mostly from insecticides. When

3 Céleres Consultancy, from 2009 to 2013, has conducted field investigations in main agricultural areas in Brazil, with a complete range of crop production. The paper only explores the question of the environmental impacts of pesticide usage.

4 The sample is not representative of cotton and soybean agriculture. The "solution" to compare conventional and GM crops results in some bias. However, the empirical strategy allows a correct comparison in terms of the incidence of pests and weeds that are the primary determinant of spraying, favoring the analysis of the effect of the adoption of GM crops.

TABLE 2 Effect of HT trait on quantity and field EIQ of herbicides and total pesticides.

	(1)	(2)	(3)	(4)
	Herbicides	**Total**	**Herbicides**	**Total**
HT Trait	0.983***	0.979***	13.685***	14.013***
	[0.084]	[0.091]	[1.545]	[1.941]
Constant	1.315***	4.756***	24.241***	85.928***
	[0.042]	[0.061]	[0.980]	[1.225]
N	182	182	182es	182
r2	0.837	0.904	0.839	0.939
Mean of Dep. Var+	1.76	3.28	30.76	82.35

Models (1) and (2): kg/ha of active ingredients of herbicides and total pesticides.
Models (3) and (4): field EIQ, for herbicides and total pesticides.
Restricted sample: farms that use both conventional and HT, seeds.
Robust standard errors in brackets.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
+Conventional seeds.

comparing this reduction with the average of 4.67 kg/ha of active ingredients of insecticides applied in fields cultivated with conventional seeds, the reduction amounts to approximately 22% of active ingredients. Relative to total pesticides, the proportional reduction amounts to 9%[5].

Consistent with the reduction in quantity of insecticides, the coefficient on EIQ indicates a reduction of 31.49 EIQ points for insecticides and 33.237 for total pesticides. To gain some perspective on this magnitude, in comparison with the general classification of active ingredients for insecticides, this is higher than the median EIQ index of 32.07. When compared to the average of 154.94 EIQ points for insecticides in fields cultivated with conventional seeds, this amounts to a reduction of 20%[6]. Hence, it can be considered a significant reduction in terms of environmental index.

Those results are consistent with the current state of the literature on environmental effects of IR seeds. Studying IR cotton seeds in India, Qaim and Zilberman (2003) found reduction of 1 kg/ha on average use of insecticides (70% compared with the baseline conventional field) while Qaim and Janvry (2005) found reductions between 1.2 kg/ha and 2.6 kg/ha of active ingredients used in Argentina, which represents about 50% reduction in comparison with conventional plots. For China, Huang et al. (2002) found even bigger reductions of about 49 kg/ha of average insecticide use (80.5% compared to the average of 60.7 kg/ha in conventional fields).

TABLE 3 OLS estimates of effects of HT trait on quantity (Kg/ha) of herbicides per toxicity level.

	(1)	(2)	(3)	(4)
	Herbicides 1	**Herbicides 2**	**Herbicides 3**	**Herbicides 4**
HT Trait	−0.083***	−0.008	0.597***	0.465***
	[0.020]	[0.051]	[0.095]	[0.087]
Constant	0.041	0.046	−0.154	1.388***
	[0.042]	[0.045]	[0.304]	[0.307]
N	180	180	180	180
r^2	0.887	0.788	0.851	0.844
Mean of Dep. Var[b]	0.23	0.22	0.78	0.51

Restricted sample: farms that use both conventional and HT, seeds.
Robust standard errors in brackets.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
+Conventional seeds.
Toxicity levels 1–4 in decreasing order (from more to less toxic). Herbicides based on Glyphosate are considered of lower toxicity level. Increases in less toxic herbicides (levels 3 and 4) are about *elevenfold* the decreases in more toxic ones (levels 1 and 2).

2.2.2 HT trait in soybean crops

Table 2 shows estimates of the effect of adoption of HT trait in soybean crops for quantities (Kg/ha) of active ingredients of herbicides and total herbicides applied (models 1 and 2) and for the field EIQ for herbicides and total pesticides (models 3 and 4). Estimates are for the restricted sample of farmers that use both conventional and IR seeds.

5 Log-linear specifications shows a decrease of 23% in the amount of insecticides and 8.8% in total quantity of pesticides. We also estimate similar models per toxicity class (I-IV in decreasing level of toxicity) which indicate reductions in all classes, the most prominent effect being for class III (medium-low level of toxicity) with a proportional decrease of 40%. Those results are available upon request.

6 The log-linear specification shows a proportional reduction of 20.1% in the EIQ index for insecticides.

The estimates show that adoption of HT trait increases the quantities (Kg/ha) of active ingredients of herbicides used by 0.983 kg/ha. For total pesticides, the coefficient is slightly smaller, indicating that the increase comes mostly from herbicides. When comparing this increase with the average of 1.76 kg/ha of active ingredients of herbicides applied in fields cultivated with conventional seeds, the increase amounts to approximately 55.8% of active ingredients. Relative to total pesticides, the proportional increase amounts to 30%.

The coefficients for the EIQ index shows that adoption of HT seeds increases the environmental impact of herbicides by 13.685 points. This represents a proportional increase of 44.4% relative to fields cultivated with conventional seeds[7]. For total pesticides the increase in the EIQ index is slightly bigger. In comparison with the general EIQ classification for herbicides, this is lower than the median value for EIQ index of 19.5. The EIQ for glyphosate is also larger than this result: 15.33. In the sample, the mean EIQ for herbicides is 37.6 and for all pesticides 89.36.

Table 3 breaks the effects on herbicides by categories of toxicity level (1–4 in decreasing order). Categories three and four show significant increases of 0.597 and 0.465 kg/ha of active ingredients respectively while categories one and two show reductions of 0.083 and 0.008 kg/ha (not statistically significant) respectively. Hence, the increase in less toxic herbicides is almost elevenfold the reduction in more toxic herbicides. This result shows that substitution among herbicides of different toxicity classes is very low, which indicates that this channel of environmental benefits is very limited. In other words, farmers adopting HT seeds are increasing the use less toxic herbicides on top of the more toxic ones. Besides weak substitution, this result also supports the idea that weed infestation is not systematically correlated with the adoption of HT seeds, which reinforces our confidence that the bias in the point estimates due to this channel might be very weak.

3 Discussion

This section provides an analysis of the environmental effects related to the use of pesticides arising from the adoption of IR cotton and HT soybean seeds. Using within-farm variation across fields treated with conventional and GM seeds, the results have shown that IR cotton reduces the number of insecticides applied to cotton crops. On the other hand, HT soybean leads to more use of herbicides. Analysis using the EIQ index shows that IR cotton reduces the environmental impact by about 20% in the treated fields compared to fields cultivated with conventional seeds. This is consistent with the previous result on Kg/ha of insecticides and confirms the environmental impact saving nature of the IR technology. The resulting environmental effects for HT soybean, on the other hand, are found to be negative. The estimates imply an increase of 36.1% on the impact of herbicides compared to fields cultivated with conventional seeds.

Regarding the quantities of herbicides of different toxicity levels, the results showed an increase in the use of lower toxicity herbicides and slight reductions for higher toxicity ones. This finding indicates very weak substitution among herbicides, which explains the higher environmental impact associated with these chemicals caused by adoption of HT soybeans.

It is worth it summing up the contributions of empirical analysis in three points. First, it contributes to uncovering environmental effects that have been hidden by the qualitative nature of the mix of herbicides induced by the HT trait. Second, ecological policymakers designing policies for biotechnology adoption might consider this new evidence to differentiate among GM traits that produce positive or negative externalities. Finally, as the composition of the EIQ index suggests, the environmental impact of pesticides can have multiple dimensions that might involve farmworker health and safety, consumer safety, and ecological effects. Hence, the results on HT soybeans point to additional avenues of work that should be taken to evaluate each of these possible channels since they can also affect other vital outcomes.

The results also suggest that previous findings on the environmental effects of HT soybeans might have been biased by the qualitative nature of the mix of herbicides[8]. Fernandez-Cornejo et al. (2002) found evidence of reduction in the use of acetamide herbicides and increase in the use of glyphosate in United States. Qaim and Traxler (2005) studying HT seeds in Argentina found a total increase of 107% in the use of herbicides, which are divided in a decreases of 87% and 100% in toxicity classes two and three, respectively, and an increase of 248% in toxicity class four. The authors suggest that this change is basically due to the use of no-till farming by adopters of HT soybeans.

Our results are not incompatible with those previous findings. In fact, we also observe a change in the composition of the mix of herbicides used towards less toxic products. This movement is predicted by the theoretical analysis that shows how the HT trait increases the value of marginal product of herbicide (glyphosate) and, therefore, the optimal amount used. On the other hand, we also find very weak substitution among herbicides of different toxicity classes, which suggests that the environmental impact of herbicides in being magnified. The analysis with the EIQ index confirms that this is not only a possibility: even inducing more use of a less toxic

7 Log-linear specifications show a proportional increase of 44.4% in the quantity of active ingredients of herbicides and 26.5% in total pesticides.

8 In fact, the National Academy of Sciences report recommends that "[be]cause of the difference in toxicity in the various chemicals used, researchers should be discouraged from publishing data that simply compare total kilograms of herbicide used per hectare per year because such data can mislead readers." (National Academies of Sciences, 2016, p. 8, p. 8).

TABLE 4 Some insights of the Adoption of GM Seed in Brazil, 2011 and 2018.

2011 Crop season

Crop	Total area (ha)	Adoption Rate (as % of Total Area including GMO Crops + Non-GMO Crops)				Area with GM Traits (Millions of Hectares)			
		IR	HT	IR/HT	Total	IR	HT	IR/HT	Total GMO
Soybean	25.0	0.0%	82.4%	0.3%	82.7%	0.0	20.6	0.07	20.7
Maize (summer + winter)	14.04	30.6%	7.5%	26.9%	65.0%	4.3	1.05	3.8	9.1
Cotton	1.55	8.5%	14.3%	16.2%	39.0%	0.132	0.222	0.251	0.605
Total Soybean + Maize + Cotton	40.6	10.9%	53.9%	10.1%	74.9%	4.4	21.9	4.1	30.4

2018 Crop Season

Crop	Total Area (ha)	Adoption rate (as % of total area including GMO crops + Non-GMO crops)				Area with GM traits (millions of hectares)			
		IR	HT	IR/HT	Total	IR	HT	IR/HT	Total GMO
Soybean	36.39	0%	40.1%	55.5%	95.6%	0.0	14.6	20.2	34.8
Maize (summer + winter)	17.3	25.4%	3.7%	59.5%	88.7%	4.4	0.646	10.3	15.3
Cotton	1.2	8.2%	14.4%	62.8%	85.4%	0.098	0.173	0.754	1.025
Total Soybean + Maize + Cotton	54.9	8.2%	28.1%	56.9%	93.2%	4.5	15.4	31.3	51.2

Source: James (2011) and ISAAA (2018).

herbicide, HT seeds cause higher environmental impact, even when controlling for the use of no-till farming.

4 The economic and environmental benefits of stacked GMOs and the opportunities generated by scientific advances in plant breeding

4.1 Stacked varieties have diffused quickly

A novelty characteristic of the last decade is the emergence of stacked genes. In 2011, stacked GMOs were cultivated in 42.2 million hectares –23.4% of the global area covered by transgenic seeds. Since then, plantations of this kind of cultivar registered strong growth, reaching 80.5 million hectares in 2018, representing 42% of the global area dedicated to GM crops.

Table 4 presents some insights that sum up the adoption situation of three main GM seeds in 2011 and 2018. These two dates are related to the studies we show in the paper: the first year is precisely in the middle of the 4-year sample to evaluate the environmental impact of IR in cotton and HT in soybean. The figures for the second year, 2018, call attention to the rapid diffusion of stacked genes that solves some caveats[9] generated by the need for growers to choose between HT and IR traits.

Inspecting Table 4, soybean, motivated by the rise in prices, contributed to pushing the GM total area. Call attention to the preference of growers for HT, performing 82.4% of the total area of soybean, with 20.6 million hectares. Despite the late approval of IR traits to corn in Brazil (in 2007), these traits performed 57.5% of the corn area in 2011. Cotton is in the last position, even with the importance of controlling bollworms.

The situation has changed sharply in 7 years. During the decade following the 2011 crop, research and development efforts in Brazil prioritized crosses between different lineages of first-generation GMOs to generate breeds able to express both the HT and IR biotech traits coming from their genitors. Stacked GMOs can be classified into four different types: 1)

9 The diffusion of stacked genes has two economic effects: a) simplify the decision process of growers related to pest and weed control, reinforcing the feature of GM seeds of reducing productive risk (Alves et al., 2020); b) contribute to the rise of seed prices via royalties (or technological fee), amplifying the menu of technological choices according to the technological level of growers (Foster and Rosenzweig, 2004).

genes that confer resistance to multiple insect species; 2) the expression of the Bt insecticidal protein in parallel with tolerance to glyphosate herbicide; 3) genetic sequences ensuring a simultaneous tolerance to different types of herbicide; 4) other types of biotech traits capable of enhancing plant tolerance to droughts and/or improving its nutritional content (Pellegrino et al., 2018).

In the Brazilian case, the great leap in the adoption of stacked cultivars started in the 2013/2014 crop with the release of Monsanto's Soja Intacta™, which expresses simultaneously both biotech traits, HT and IR. In a mere 5 years, Intacta™ 's cultivation area went from 2.3 million hectares in 2013 to 20.2 million hectares in 2018, making this cultivar the GMO with the largest diffusion during the 2010s (ISAAA, 2018). In the face of the increasing replacement of soybean varieties which express only tolerance to glyphosate by Intacta™, the HT + IR seeds became predominant in the Brazilian soy culture. Moreover, Table 4 reveals that Brazilian GM maize and cotton crops experienced a similar situation, increasingly favoring stacked GMOs with respect to the first generation ones.

The revealed preference for stacked genes calls attention to the importance of integrating the modules that compose the grain production. It means that from soil preparation to harvesting, the combination of GM traits facilitates crop management and reduces risks associated with critical delays in the sowing period (Carauta et al., 2017). The use of stacked genes forcibly reduces the GGE emissions by eliminating some tasks in soil preparation, sowing, and pest control and provides a kind of insurance to growers once the plant is resistant to essential pests (Alves et al., 2020).

In the section dedicated to evaluating the environmental impact of GM seeds, we use a unique farm-level dataset documenting the adoption of GM seeds between 2009 and 2013 by commercial farms in Brazil. Table 4 suggests that data of the soybean, maize and cotton plantations Environmental Impact Index encompass a period characterized by an ample predominance of first generation GMO cultivation. Since then, the adoption of stacked GMOs has registered a strong growth, reaching 31.1 million hectares in 2018 (60.94% of the of the Brazilian crop area dedicated to transgenic seeds).

The fast pace of diffusion of stacked GMOs in Brazil and worldwide[10] has motivated a variety of studies about the economic and environmental impact of this technological innovation. These works point to the gain in agricultural productivity, the farmers' increasing profits, the decrease in the use of crop protection chemicals, and the reduction of carbon emissions, as the main benefits of stacked seeds compared with single-trait biotech GMOs (Waquil et al., 2013; Pellegrino et al., 2018; Brookes and Barfoot, 2020).

In a meta-analysis published in Nature Scientific Reports, Pellegrino et al. (2018) reviewed 76 scientific publications in order to analyze the economic impact of four types of GM maize seeds[11]. The authors determined that the decrease in pesticide application and the increase in crop yield were more significant in the areas planted with quadruple stacked hybrids. The authors have found that the stacking of genes has been successful in widening full protection against pests and delaying the appearance of insects resistant to the applications of agricultural biotechnology.

Studies comparing HT + IR soybean seeds with single-trait biotech cultivars of the same grain obtained similar results to the ones found in the case of maize. According to Brookes and Barfoot (2020), the adoption of Soja Intacta™ provided to South American growers economic benefits equivalent to 10.2 billion dollar during 2013–2018. This implies that for every US$ 1 invested in Intacta™ technology, the growers received approximately US$ 3.88 of additional profit. This economic gain was the result of a production increase of 27.3 million tons of soybean (considering the productivity increases obtained from a total cultivated area of 73.6 million hectares during 6 years) and the expense reduction in weed and pest control.

Intacta ™ soybean cultivation reduced chemical protection application in such magnitude as to imply a fall in the Environmental Impact Quotient (EIQ) of GM soybean crops:

"Intacta soybeans have enabled soybean growers to reduce the average number of insecticide treatments by about 4 (from an average of 8–10 sprays on conventional or GM HT only crops) in Brazil [. . .] Based on these savings, in 2018, the use of this technology resulted in a reduction of four million kg of insecticide active ingredient use, equal to 13.1% of total insecticide used on the soybean crops in the four countries. The EIQ saving in 2018 was equal to −13.8%. Over the 6 years, the total insecticide active ingredient usage saving has been 14.9 million kg (−8.2%) and the associated environmental impact, as measured by the EIQ indicator fell by 8.6% (Brookes and Barfoot, 2020, p.98-99)."

The authors also highlighted that the adoption of Soja Intacta ™ has reduced the level of greenhouse gas emissions associated with soybean cultivation. This is mainly due to fuel savings caused by the reduction by half of aerial spraying in areas planted with HT seeds or traditional varieties.

10 In 2011, stacked GMOs were cultivated in 42.2 million hectares, −23.4% of the global area covered by transgenic seeds. Since then, plantations of this kind of cultivar registered a strong growth, reaching 80.5 million hectares in 2018, representing 42% of the global area dedicated to GM crops.

11 The comparison involved the following hybrid corn: i) GM single-trait biotech seeds (lepidoptera resistance); ii) double stack (lepidoptera resistance + glufosinate tolerance); iii) triple stack (lepidoptera resistance + coleoptera resistance + glufosinate tolerance); iv) quadruple stack (lepidoptera resistance + coleoptera resistance + glufosinate/glyphosate tolerance).

Furthermore, by eliminating unwanted and competing plants, Intacta™ technology facilitates the transition from traditional planting systems (predominant in non-transgenic seed cultivation) to direct planting systems, far less dependent on soil preparation operations, such as mechanized plowing (Brookes and Barfoot, 2018). For these reasons, after 5 years of its adoption, Intacta ™ technology contributed to a carbon dioxide emission reduction equivalent to the removal of 3.3 million cars from the roads (ISAAA, 2018).

4.2 Limitations of gene stacking techniques and future implications of the new genome editing technologies

Despite the advantages provided by stacked GMOs for pest control and reduction of greenhouse gas emissions, some researchers warn about the difficulties the seed industry has faced to adapt itself to climate change, specially, in abiotic stress situations. Graff et al. (2009) raised the hypothesis that the pace of development of seeds that need less water for growing fell short of what would be expected. Throughout the decade following this study, the diffusion of biotech traits capable of increasing drought tolerance in plants has also been slow[12].

A climatic event in Argentina elucidated one of the main challenges the seed industry will face in the following decades. During the 2018/2019 crop season, "a severe drought during the peak summer months reduced the area planted to biotech soybean" (ISAAA, 2018, p. 18–19), which led to a reduction of production and put in evidence a considerable limitation, inherent to the cross-hybridization techniques used in the development of stacked GMOs.

The stacking of many biotech traits tends to compromise the myriad of other agronomic attributes not controlled by the transferred genes, which can ultimately reduce the physiological quality and productivity of the host plant. If on one hand, the technical limitations of transgenic processes and gene stacking has hindered the diffusion of new agronomic traits (Qaim, 2020), on the other, various authors are hopeful that new genome editing techniques based on CRISPR-Cas9 can, in the future, alleviate the above technological obstacles (Vats et al., 2019; Zaidi et al., 2019).

Genome editing techniques are already being used to develop tolerance to abiotic stress in soybean, maize, rice, wheat, and bean cultivars, as well as in several other cultivations. Therefore, there exists an expectation that the CRISPR-Cas9 system revolutionizes the development process of agronomic traits, enabling the expression of a much larger number of traits than the ones currently observed in the GMOs existent in the market (Vats et al., 2019; Zaidi et al., 2019; IHS Markit, 2020; Qaim, 2020)[13].

Even though gene-edited seeds are still not used at a commercial scale and, up to this moment, their economic and environmental impact cannot be observed nor quantified (Qaim, 2020), it is already possible to point out the main technology holders of the most important editing technologies as well as to indicate some cultivars already approved by North American and Brazilian regulatory authorities.

According to Egelie et al. (2016, p.1028), the Boston academic cluster (consisting of the Broad Institute, MIT and Harvard University) and the University of California, Berkeley jointly concentrate proprietary control of the main components of the CRISPR-Cas9 system. The cluster was responsible for 20% of the patents filed in this field up to 2016 (131 documents). The University of California owned a smaller portfolio, with 14 patent families which, however, included some of the essential enabling technologies for the whole system. In this way, such institutions held full control of the medical applications of the CRISPR-Cas9 technology. On the other hand, the control of agricultural and food applications of the same technology was distributed in a more balanced way in the corporate sector, with Dow-DuPont playing a prominent role.

The work of Egelie et al. (2016) is crucial to understand the uncertainty involving the CRISPR system at that moment. The first great uncertainty involved the property of the Cas9 molecular scissors. The Boston cluster and the University of California, Berkeley filed, almost simultaneously, patents claiming the discovery. The USPTO granted the ownership of the enzyme to the Boston cluster. Soon after, the University of Berkeley filed a request for patent revocation to the same agency. In spite of this conflict, both academic groups created their own startups. Caribou Biosciences is a commercial spin-off of the University of California, Berkeley, in the same way that Editas Medicine was created by the MIT/Broad Institute.

In both cases, the startups were granted exclusive patent licenses for commercializing the biotechnologies developed by the original universities, so companies that decide to use the

12 For instance, the United States planted 33.14 million hectares with transgenic maize seeds in 2018. In the same year, only two million hectares were planted with GMOs with a drought resistant gene stacked in their genome (ISAAA, 2018).

13 The term CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) refers to a natural defense system bacteria use against viral infections. When infected, bacteria synthesize enzymes (the most famous of them is called Cas9) which act as molecular scissors able to cut the virus DNA and store some fragments in their own genome. This procedure makes it possible that, in case of future infections, a bacteria recognizes the virus and destroys it (Pausch et al., 2020). The winners of the Nobel Prize for Chemistry in 2020, Jennifer Doudna and Emmanuelle Charpentier, showed that the Cas9 enzyme can be guided by the instructions of a messenger RNA molecule to recognize and cut DNA sequences of different types of organisms. With the cleavage, the gene is disabled, and, during its repair, the cut parts can be edited to correct genetic mutations or, in the specific case of the seed industry, to codify new useful functionalities in plants for agricultural activities (Zhang et al., 2019).

Cas9 tool would inevitably have to negotiate sublicenses with one or both of the above startups. Some statements from Caribou and Editas led Egelie et al. (2016) to fear that both startups would opt for the internal development of new products instead of transferring the technologies to the seed industry, which could ultimately create legal obstacles for the development of new agricultural applications of CRIPSR-Cas9.

Fortunately, this pessimistic scenario did not materialize. As Zhang et al. (2019) highlighted, the scientific community identified other molecular scissors (*e.g.* Cas12a, Cas13a, CasX, etc.) capable of replacing Cas9 in the CRISPR system. Furthermore, the lead companies in the seed industry did not have much difficulty negotiating technological licenses with commercial representatives of both academic groups disputing the ownership of the Cas9 enzyme (IHS Markit, 2020).

For instance, Dow-DuPont was one of the first companies to negotiate a technological sublicense for the purpose of exploring Caribou Biosciences agricultural technologies (Egelie et al., 2016), which later was inherited by Corteva (a spin-off from this conglomerate which become a standalone company). More recently, Corteva negotiated a number of tripartite agreements of intellectual property which involved, at the same time, the academic institutions composing the Boston cluster and several bioinformatic companies, such as the J. R. Simplot Company, Yield10 Bioscience and Amfora (IHS Markit, p.2020). These tripartite agreements established legal conditions for the utilization of the molecular scissors developed by MIT and Harvard to do genome-editing of Corteva's cultivars.

By virtue of these joint research efforts, Corteva obtained its first gene-edited cultivar, namely, the waxy corn hybrids (hybrid corn with waxy starch). In short, Corteva's scientists disabled the amylose gene with the intention of raising the level of amylopectin in corn starch, thus benefiting the frozen food, dye and glue sectors. The waxy corn hybrids received the approval of the United States Department of Agriculture (USDA) in 18 April 2016 (see Chart 1), going down in history as the second cultivar developed from the CRISPR system to be released for planting and commercialized in the United States. Since then, approval events have only multiplied in that country.

The approval timetable transcribed in Chart 1 has influenced the Brazilian regulatory authorities. On 15 January 2018, the National Biosecurity Technical Committee (CTNBio) from Brazil enacted the Normative Resolution n°16 (RN16) establishing regulatory parameters for the gene-editing technologies. The RN16 resolutions follow the USDA positioning, namely that the requests should be analyzed on a case-by-case basis according to the method of production of the cultivar. It follows that the existence or not of DNA sequences coming from other species represents the main criterion to differentiate the GMOs from gene-edited cultivars. In the absence of exogenous DNA fragments and/or other applications of recombinant DNA technology, the varieties developed through the CRISPR system should be considered as non-transgenic conventional organisms (Eriksson et al., 2019).

CHART 1 Gene-edited cultivars approved by the United States Department of Agriculture (USDA).

Approval date	Crop	Agronomic trait
04/13/2016	Mushroom	Do not turn black on the cut
04/18/2016	Maize	Increase in Amylopectin levels
11/15/2016	Potato	Do not turn black on the cut
12/02/2016	Potato	Do not turn black on the cut
08/29/2017	False flax	Increase in Omega-3 levels
10/16/2017	Soybean	Salt and drought resistance
11/25/2017	Alfalfa	Enhancement of digestibility
01/12/2018	Maize	Fungal resistance
03/19/2018	Maize	Productivity enhancement
03/20/2018	Wheat	Higher fiber content
05/14/2018	Tomato	Improvement of the harvesting process
08/06/2018	Pennycress	Improvement of oil quality
11/07/2018	False flax	Increase in Omega-3 levels

Source: USDA, adapted from (Venâncio, 2019, p.31).

In view of the alignment between the RN16 and the North American regulatory framework, the request for regulation of the waxy corn hybrids in Brazil made by Corteva happened quickly. In a polling that took place in November 2018, the CTNBio granted to waxy corn hybrids the condition of conventional organisms, becoming one of the first gene-edited cultivars in Brazilian national territory (Eriksson et al., 2019). Very recently, in a CTNBio meeting on 9 December 2021, the Committee approved the first edited sugarcane cultivars in the world. The Cana Flex 1 (enhancement of the digestibility of cell walls) and the Cana Flex 2 (higher levels of sucrose) were developed by the EMBRAPA Agroenergia to facilitate the production of first and second generation ethanol as well as the manufacture of other bioproducts from sugarcane bagasse.

One of the main criticisms aimed at GMOs is related to the concentration of the R&D efforts on just four products with strong commercial appeal–GM maize, soybean, cotton, and canola seeds. Therefore, the vast majority of agricultural crops seem to have become orphan from the productivity gains derived from the application of recombinant DNA technology in agriculture (Graff et al., 2009). Add to this criticism, another one equally relevant, questioning the seed industry focusing on only two biotech traits: HT and IR (Ferrari et al., 2021). When compared with other already released transgenic events, the requests of approval of gene-edited cultivars made in the United States (Chart 1) and in Brazil seem to indicate a much greater balance: 1) between the agricultural crops that could be considered by the new biotechnological advances, and 2) regarding to the range of agronomic traits that might be included in the research and genetic improvement programs.

5 Conclusion

GM crops have diffused quickly since 1996, focused on three platform crops and canola, restricted to a few countries, and two main traits: insect resistance and herbicide tolerance. Despite the restrictions, GM varieties were adopted by the more prominent producers and exporters in the world, notably the United States, Brazil, Canada, Argentina, and India (more than 90% of the total GM adopted).

The contrast between "lovers" and "haters" of GM crops has spurred studies to evaluate impacts. Economic gains of GM adoption are not easy to assess once HT varieties are not related to cost reduction; the two main reasons for adopting HT varieties are risk reduction and the simplification of production processes. However, these factors allow the increase in land productivity. Using IR varieties contributes to cost and risk reduction and simplifies the productive processes. Still, it can induce the substitution of pesticides due to the appearance of new and more resistant pests. All these considerations are based on the literature.

Profiting from the unique opportunity to analyze data from a 5-year research field from 2009 to 2013, the paper tests two hypotheses related to IR and HT varieties, using the most paradigmatic crops: cotton in the IR case and soybean in HT. Results from IR are straightforward and adhere to the results verified in the literature: the IR trait reduces the environmental impact by about 20% compared to crops using conventional seeds. There is a reduction in the quantity per hectare of insecticides usage, but more importantly, the GM seeds reduce the impact by using 22% less pesticide. It also contributes to substitute the pesticide usage in 9%, meaning that it is more challenging to replace insecticides in cotton. The substitution effect between pesticides was, in this case, less significant than reduction, so both estimates, quantities, and EIQ have pointed to a positive environmental contribution of GM adoption.

A different scenery was seen in the case of HT adoption in soybeans. In this case, the evaluation based on EIQ indexes has shown to be relevant to answering the research questions proposed. The GM production system used 55.8% and 30% more active ingredients than the conventional system in herbicides and total pesticides. Since glyphosate (the leading herbicide in the GM system) is less toxic than others used in the conventional method, there was room for the substitution effect. The increase in the EIQ index for herbicides is 44.4% and 26.5% of total pesticides, which is quite disappointing. The substitution effect from more toxic (1 and 2 categories) to less harmful was not enough to reduce the environmental damage of the GM system to weed control in soybean in Brazil. The choice of GM seeds has generated managerial advantages and possibilities to intensify land usage (no-till, double cropping) with the side effect that weed infestations are not systematically correlated with adopting HT seeds.

Going beyond the conclusion that the use of the EIQ index is relevant to understanding the environmental impacts of GM in Brazil is the fact that the combination of IR + HT can make GM technology more favorable. The diffusion direction was to adopt stacked GM seeds that avoid a choice between being efficient in weed control and pollutant in controlling pests. Data shows a sharp change in the adoption of GM. In 2011, HT was integrally adopted by whom had GM as a choice. The adoption rate of GM in cotton was low. In 2018, the higher level (more than double) of GM was due to HT + IT traits (63% of the varieties).

Although the diffusion processes have been technology-led, the quick response of the seed industry (the fierce rivalry between innovative firms is still in place) shows the attention to growers' demands and the incentives the technological fees provided to leading firms. The recent investigation of the frontier of plant breeding points to the diversification of traits and cultures, allowing the technology to contribute to problems related to global warming effects in agriculture and overpass the criticism coming from grassroots movements and the people with an urban view of agriculture.

Data availability statement

The raw data supporting the conclusion of this article will be made available under request to the corresponding author.

Author contributions

RN: paper strategy, empirical approach and analysis, econometric estimation; JS: contextualization, literature review, paper strategy, conclusion; VF: future scenery plant breeding review, diffusion aspectes, paper organizatons, lilterature review.

Acknowledgments

Céleres Consultancy contributed gently by allowing the access to the data bank used in section 2 of the paper. The results of the project "Evaluation of Social-economic and environmental impacts on the diffusion of Bt cotton varieties in Brazilian cotton growers communities", Latin American and Caribeean Biosafety project (Embrapa/LAC Biosafety) contributed to the conceptual part of the paper. Financial support from CAPES/Fulbright PhD fellowship (grant 2256-08-8) is greatly acknowledged.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Alves, L. R. A., Filho, J. B. D. S. F., Silveira, J. M. F. J. D., Costa, M. S. D., Osaki, M., Lima, F. F. D., et al. (2020). Genetically modified corn adoption in Brazil, costs and production strategy: Results from a four-year field survey. *Rev. Econ. E Agronegócio* 18, 1–23. doi:10.25070/rea.v18i3.11083

Barrows, G., Sexton, S., and Zilberman, D. (2014). Agricultural biotechnology: The promise and prospects of genetically modified crops. *J. Econ. Perspect.* 28, 99–120. doi:10.1257/jep.28.1.99

Bonny, S. (2016). Genetically modified herbicide-tolerant crops, weeds, and herbicides: Overview and impact. *Environ. Manage.* 57, 31–48. doi:10.1007/s00267-015-0589-7

Brookes, G., and Barfoot, P. (2018). Environmental impacts of genetically modified (GM) crop use 1996-2016: Impacts on pesticide use and carbon emissions. *Gm. Crops Food* 9, 109–139. doi:10.1080/21645698.2018.1476792

Brookes, G., and Barfoot, P. (2020). *GM crops: Global socio-economic and environmental impacts 1996-2018*. Dorchester, UK: PG Economics Ltd.

Carauta, M., Libera, A. A. D., Hampf, A., Chen, R. F. F., Silveira, J. M. F. J., and Berger, T. (2017). On-farm trade-offs for optimal agricultural practices in mato grosso, Brazil. *Rev. Econ. E Agronegócio* 15. doi:10.25070/rea.v15i3.505

Carpenter, J. E. (2010). Peer-reviewed surveys indicate positive impact of commercialized GM crops. *Nat. Biotechnol.* 28, 319–321. doi:10.1038/nbt0410-319

Carpenter, J., and Gianessi, L. (1999). Herbicide tolerant soybeans: Why growers are adopting Roundup Ready varieties. *AgBioforum* 2 (2), 65–72.

Crost, B., and Shankar, B. (2008). Bt-cotton and production risk: Panel data estimates. *Int. J. Biotechnol.* 10, 122–131. doi:10.1504/IJBT.2008.018349

Datta, S., Dhillon, B. S., Gautam, P. L., Karihaloo, J. L., Mahadevappa, M., Mayee, C. D., et al. (2019). India needs genetic modification technology in agriculture. doi:10.18520/cs/v117/i3/390-394

Dias, M., Rocha, Rocha R., and Soares, R. R. (2019). *Glyphosate use in agriculture and birth outcomes of surrounding populations*. Bonn, Germany: IZA Institute of Labor [Preprint]. IZA Discussion Papers 12164 Available at: https://www.econstor.eu/bitstream/10419/196662/1/dp12164.pdf.

Egelie, K. J., Graff, G. D., Strand, S. P., and Johansen, B. (2016). The emerging patent landscape of CRISPR–Cas gene editing technology. *Nat. Biotechnol.* 34, 1025–1031. doi:10.1038/nbt.3692

Eriksson, D., Kershen, D., Nepomuceno, A., Pogson, B. J., Prieto, H., Purnhagen, K., et al. (2019). A comparison of the EU regulatory approach to directed mutagenesis with that of other jurisdictions, consequences for international trade and potential steps forward. *New Phytol.* 222, 1673–1684. doi:10.1111/nph.15627

Fernandez-Cornejo, J., Klotz-Ingram, C., and Jans, S. (2002). Farm-Level Effects of Adopting Herbicide-Tolerant Soybeans in the U.S.A. *J. Agric. Appl. Econ.* 34, 149–163. doi:10.1017/S1074070800002200

Ferrari, V. E., Silveira, J. M. F. J. da, and Dal-Poz, M. E. S. (2021). Patent network analysis in agriculture: a case study of the development and protection of biotechnologies. *Econ. Innovation New Technol.* 30, 111–133. doi:10.1080/10438599.2019.1684645

Foster, A. D., and Rosenzweig, M. R. (2004). Agricultural Productivity Growth, Rural Economic Diversity, and Economic Reforms: India, 1970–2000. *Econ. Dev. Cult. Change* 52, 509–542. doi:10.1086/420968

Graff, G. D., Hochman, G., and Zilberman, D. (2009). The Political Economy of Agricultural Biotechnology Policies. *AgBioForum* 12 (1), 34–46.

Graff, G. D., Rausser, G. C., and Small, A. A. (2003). Agricultural Biotechnology's Complementary Intellectual Assets. *Rev. Econ. Stat.* 85, 349–363. doi:10.1162/003465303765299864

Huang, J., Hu, R., Rozelle, S., Qiao, F., and Pray, C. E. (2002). Transgenic varieties and productivity of smallholder cotton farmers in China. *Aust. J. Agric. Resour. Econ.* 46, 367–387. doi:10.1111/1467-8489.00184

IHS Markit (2020). *Agrow - game changers gene-editing technologies and their applications*. London, United Kingdom: Copyright HIS Markit All Rights Reserved.

ISAAA (2018). *Global status of commercialized biotech/GM crops in 2018: Biotech crops continue to help meet the challenges of increased population and climate change*. Ithaca, NY: ISAAA Brief No, 54.

James, C. (2011). *Global status of commercialized biotech/GM crops: 2011*. Ithaca, NY: ISAAA Brief No, 43.

Kalaitzandonakes, N., Lusk, J., and Magnier, A. (2018). The price of non-genetically modified (non-GM) food. *Food Policy* 78, 38–50. doi:10.1016/j.foodpol.2018.02.005

Kathage, J., and Qaim, M. (2012). Economic impacts and impact dynamics of Bt (Bacillus thuringiensis) cotton in India. *Proc. Natl. Acad. Sci. U. S. A.* 109, 11652–11656. doi:10.1073/pnas.1203647109

Klümper, W., and Qaim, M. (2014). A Meta-Analysis of the Impacts of Genetically Modified Crops. *PLOS ONE* 9, e111629. doi:10.1371/journal.pone.0111629

Kovach, J., Petzoldt, C., Degni, J., and Tette, J. (1992). A Method to Measure the Environmental Impact of Pesticides. *New York's Food Life Sci. Bull.* 139, 1–8.

Kranthi, K. R., and Stone, G. D. (2020). Long-term impacts of Bt cotton in India. *Nat. Plants* 6, 188–196. doi:10.1038/s41477-020-0615-5

Krishna, V., Qaim, M., and Zilberman, D. (2016). Transgenic crops, production risk and agrobiodiversity. *Eur. Rev. Agric. Econ.* 43, 137–164. doi:10.1093/erae/jbv012

Lamichhane, J. R., Devos, Y., Beckie, H. J., Owen, M. D. K., Tillie, P., Messéan, A., et al. (2017). Integrated weed management systems with herbicide-tolerant crops in the European Union: lessons learnt from home and abroad. *Crit. Rev. Biotechnol.* 37, 459–475. doi:10.1080/07388551.2016.1180588

Maia, A. G., and Silveira, J. M. F. J. (2016). How Latent Attitudes Affect Farmers' Preferences for Genetically Modified Seeds: The Case of Small Corn Growers in Brazil. *AgBioForum* 19 (1), 72–84.

National Academies of Sciences (2016). *Genetically engineered crops: Experiences and prospects*. Washington, DC: The National Academies Press. doi:10.17226/23395Engineering, and Medicine

Oliveira, P. R. S., Silveira, J. M. F. J. da, and Bullock, D. S. (2020). Innovation in GMOs, technological gap, demand lag, and trade. *Agribusiness* 36, 37–58. doi:10.1002/agr.21622

Pausch, P., Al-Shayeb, B., Bisom-Rapp, E., Tsuchida, C. A., Li, Z., Cress, B. F., et al. (2020). CRISPR-CasΦ from huge phages is a hypercompact genome editor. *Science* 369, 333–337. doi:10.1126/science.abb1400

Pellegrino, E., Bedini, S., Nuti, M., and Ercoli, L. (2018). Impact of genetically engineered maize on agronomic, environmental and toxicological traits: a meta-analysis of 21 years of field data. *Sci. Rep.* 8, 3113. doi:10.1038/s41598-018-21284-2

Punt, M. J., and Wesseler, J. (2018). The Formation of GM-free and GM Coasean Clubs: Will They Form and If So How Much Can They Achieve? *J. Agric. Econ.* 69, 413–438. doi:10.1111/1477-9552.12235

Qaim, M., and Janvry, A. D. (2005). Bt cotton and pesticide use in Argentina: economic and environmental effects. *Environ. Dev. Econ.* 10, 179–200. doi:10.1017/S1355770X04001883

Qaim, M. (2020). Role of New Plant Breeding Technologies for Food Security and Sustainable Agricultural Development. *Appl. Econ. Perspect. Policy* 42, 129–150. doi:10.1002/aepp.13044

Qaim, M. (2009). The Economics of Genetically Modified Crops. *Annu. Rev. Resour. Econ.* 1, 665–694. doi:10.1146/annurev.resource.050708.144203

Qaim, M., and Traxler, G. (2005). Roundup Ready soybeans in Argentina: farm level and aggregate welfare effects. *Agric. Econ.* 32, 73–86. doi:10.1111/j.0169-5150.2005.00006.x

Qaim, M., and Zilberman, D. (2003). Yield Effects of Genetically Modified Crops in Developing Countries. *Science* 299, 900–902. doi:10.1126/science.1080609

Qiao, F. (2015). Fifteen Years of Bt Cotton in China: The Economic Impact and its Dynamics. *World Dev.* 70, 177–185. doi:10.1016/j.worlddev.2015.01.011

Schütte, G., Eckerstorfer, M., Rastelli, V., Reichenbecher, W., Restrepo-Vassalli, S., Ruohonen-Lehto, M., et al. (2017). Herbicide resistance and biodiversity: agronomic and environmental aspects of genetically modified herbicide-resistant plants. *Environ. Sci. Eur.* 29, 5. doi:10.1186/s12302-016-0100-y

Sexton, S., and Zilberman, D. (2012). "Land for Food and Fuel Production: The Role of Agricultural Biotechnology," in *The intended and unintended effects of U.S. Agricultural and biotechnology policies*. Editors J. S. G. Zivin and J. M. Perloff (Chicago: University of Chicago Press), 269–288.

Smale, M., Zambrano, P., Paz-Ybarnegaray, R., and Fernández-Montaño, W. (2012). A Case of Resistance: Herbicide-tolerant Soybeans in Bolivia. *AgBioForum* 15 (2), 191–205.

Smyth, S. J., Phillips, P. W. B., and Kerr, W. A. (2015). Food security and the evaluation of risk. *Glob. Food Sec.* 4, 16–23. doi:10.1016/j.gfs.2014.08.001

Tabashnik, B. E., and Carrière, Y. (2017). Surge in insect resistance to transgenic crops and prospects for sustainability. *Nat. Biotechnol.* 35, 926–935. doi:10.1038/nbt.3974

Vats, S., Kumawat, S., Kumar, V., Patil, G. B., Joshi, T., Sonah, H., et al. (2019). Genome Editing in Plants: Exploration of Technological Advancements and Challenges. *Cells* 8, 1386. doi:10.3390/cells8111386

Veettil, P. C., Krishna, V. V., and Qaim, M. (2017). Ecosystem impacts of pesticide reductions through Bt cotton adoption. *Aust. J. Agric. Resour. Econ.* 61, 115–134. doi:10.1111/1467-8489.12171

Venâncio, R. (2019). CRISPR, a sigla do futuro do agro. *Plant Proj.* 13, 20–33.

Waquil, J. M., Dourado, P. M., Carvalho, R. A. de, Oliveira, W. S., Berger, G. U., Head, G. P., et al. (2013). Manejo de lepidópteros-praga na cultura do milho com o evento Bt piramidado Cry1A.105 e Cry2Ab2. *Pesq. Agropec. Bras.* 48, 1529–1537. doi:10.1590/S0100-204X2013001200001

Zaidi, S. S.-A., Vanderschuren, H., Qaim, M., Mahfouz, M. M., Kohli, A., Mansoor, S., et al. (2019). New plant breeding technologies for food security. *Science* 363, 1390–1391. doi:10.1126/science.aav6316

Zhang, Y., Malzahn, A. A., Sretenovic, S., and Qi, Y. (2019). The emerging and uncultivated potential of CRISPR technology in plant science. *Nat. Plants* 5, 778–794. doi:10.1038/s41477-019-0461-5

frontiers | Frontiers in Bioengineering and Biotechnology

TYPE Policy and Practice Reviews
PUBLISHED 31 August 2022
DOI 10.3389/fbioe.2022.971402

OPEN ACCESS

EDITED BY
Patricia Machado Bueno Fernadea,
Federal University of Espirito Santo,
Brazil

REVIEWED BY
Hector Quemada,
Retired, United States
Jitesh Kumar,
University of Minnesota Twin Cities,
United States

*CORRESPONDENCE
Joshua G. Philips,
Joshua.Philips@health.gov.au
Andrea V. Robold,
Andrea.Robold@health.gov.au

SPECIALTY SECTION
This article was submitted to Biosafety and Biosecurity,
a section of the journal
Frontiers in Bioengineering and Biotechnology

RECEIVED 17 June 2022
ACCEPTED 05 August 2022
PUBLISHED 31 August 2022

CITATION
Philips JG, Martin-Avila E and Robold AV (2022), Horizontal gene transfer from genetically modified plants - Regulatory considerations.
Front. Bioeng. Biotechnol. 10:971402.
doi: 10.3389/fbioe.2022.971402

Horizontal gene transfer from genetically modified plants - Regulatory considerations

Joshua G. Philips*, Elena Martin-Avila and Andrea V. Robold*

Office of the Gene Technology Regulator, Canberra, ACT, Australia

Gene technology regulators receive applications seeking permission for the environmental release of genetically modified (GM) plants, many of which possess beneficial traits such as improved production, enhanced nutrition and resistance to drought, pests and diseases. The regulators must assess the risks to human and animal health and to the environment from releasing these GM plants. One such consideration, of many, is the likelihood and potential consequence of the introduced or modified DNA being transferred to other organisms, including people. While such gene transfer is most likely to occur to sexually compatible relatives (vertical gene transfer), horizontal gene transfer (HGT), which is the acquisition of genetic material that has not been inherited from a parent, is also a possibility considered during these assessments. Advances in HGT detection, aided by next generation sequencing, have demonstrated that HGT occurrence may have been previously underestimated. In this review, we provide updated evidence on the likelihood, factors and the barriers for the introduced or modified DNA in GM plants to be horizontally transferred into a variety of recipients. We present the legislation and frameworks the Australian Gene Technology Regulator adheres to with respect to the consideration of risks posed by HGT. Such a perspective may generally be applicable to regulators in other jurisdictions as well as to commercial and research organisations who develop GM plants.

KEYWORDS

horizontal gene transfer, lateral gene transfer, vertical gene transfer, GM plants, GMO risk analysis, gene technology regulation

1 Introduction

Horizontal or lateral gene transfer (HGT) is the stable and heritable acquisition by an organism, of genetic material that did not originate from a parental donor (Keese, 2008). Any DNA sequence, including endogenous sequences or foreign DNA introduced into a genetically modified (GM) organism, has the potential to undergo HGT. This potential is only fulfilled when the genetic material stably integrates into the genome of the recipient and is then transmitted to its offspring (Hülter and Wackernagel, 2008; Brigulla and Wackernagel, 2010; Huang, 2013). HGT can benefit the recipient by enabling the acquisition of a beneficial pre-existing trait from another organism, regardless of

phylogenetic distance. It thereby, like vertical gene transfer, accelerates evolution (Fournier et al., 2015).

In Australia, the Gene Technology Regulator (the Regulator) receives applications for the intentional environmental release of GM plants and, as part of the assessment process of these applications, must consider the risks to human and animal health and to the environment from gene technology posed by the proposed activities. GM plants may have genetic elements sourced from other organisms imparting desired traits, e.g., increased nutritional value; drought, pest and disease resistance; or increased productivity. While gene transfer is most likely to occur to sexually compatible relatives through vertical gene transfer, the likelihood of gene transfer to non-sexually compatible organisms *via* HGT also needs to be considered as part of the risk assessment.

Similarly, in Europe, the Commission Regulation (EU) 503/2013 of 3 April 2013 (on applications for authorisation of genetically modified food and feed) states that "The applicant shall assess the probability of horizontal gene transfer from the product to humans, animals and microorganisms and any potential associated risk when intact and functional nucleic acid(s) remains in the genetically modified food and feed." In the United Kingdom, the independent Advisory Committee of Releases into the Environment (ACRE) also considers HGT in their assessment for application for the release of genetically modified organisms (GMOs) (ACRE, 2013). Other regulatory authorities may also need to consider HGT before issuing an authorisation or licence. In this review, we discuss the recent advances in detecting HGT events and present updated evidence of the likelihood, factors, barriers and pathways for HGT to take place from GM plants to a variety of other organisms.

2 Legislative context and risk analysis applicable to considering risks imposed by HGT

In Australia, Regulations 9A and 10 of the Gene Technology Regulations 2001 (OGTR, 2020) specify the risks and matters that must be considered in the risk assessment for an environmental release of GMOs (Figure 1). Considerations relating to gene flow are 1) the potential for spread and persistence of a GMO's genetic material in the environment and 2) the potential of the GMO to transfer genetic material to another organism. The risk assessment seeks to evaluate the level of risk from the activities with a GMO if HGT from the GMO into other organisms was successful, compared to the status quo.

In Australia, the Risk Analysis Framework (RAF) (OGTR, 2013), in accordance with Australia's *Gene Technology Act 2000* and Regulations, outlines the approach that the Regulator takes to conduct the risk assessment of proposed activities with a GMO. Such activities include the proposed environmental release of the GMO. The RAF describes the three essential components needed for a scenario (set of circumstances) that might give rise to harm as a result of activities conducted with the GMO. The three components are: 1) a source of potential harm, which may be a new or altered property/trait of the GMO; 2) a potential harm to people or the environment; and 3) a plausible causal linkage between components 1) and 2). If a plausible causal linkage or potential harm cannot be described, then the source of potential harm poses no risk (OGTR, 2013).

While HGT per se is not considered a risk, it fits into the pathway component of risk as it can link the introduced or modified DNA to a potential harm to people or the environment. Therefore, the likelihood of HGT occurring determines the potential for any harm. As the number of steps in a pathway leading to harm increases, the likelihood of harm occurring decreases. The Regulator considers the likelihood of occurrence of HGT and the severity of adverse outcomes if HGT was successful. If the level of risk is increased compared to the status quo, then the Regulator may include specific risk management measures to interrupt steps in a pathway and reduce the likelihood of harm occurring or refuse to approve the proposed intentional environmental release. For example, as a measure to limit the likelihood of gene flow by vertical gene transfer from GM plants, an exclusion zone can be imposed where sexually compatible plants are not permitted to be grown.

It should also be noted that risk analysis on a proposal for the release of GM plants occurs in the context of the receiving environment. For example, if a GM (transgenic) DNA sequence was sourced from a ubiquitous bacteria or fungi, then this forms part of the context as the DNA sequence is already in the environment. If HGT from the bacteria or fungi is more likely than HGT from the proposed activities with the GM plant, then the potential of HGT resulting in harm from the release of the GM plant can be no greater than the risk from the parent organism. Similarly, the likelihood of harm occurring as a result of the GM plant release is compared to that of harm occurring in the absence of the genetic modification, i.e., the non-GM plant.

3 Advances and limitations of new HGT detection methods

Until recently, comparative analysis for detection of HGT events relied on very limited databases of manually annotated genes (Dupont and Cox, 2017). However, the expansion of next generation sequencing has allowed an ever-increasing number of whole genomes from a vast range of species to be readily available for multiple genome comparisons. As a result, detection of HGT events for all annotated genes can now be performed bioinformatically. By using this approach, HGT can be inferred by either the parametric or phylogenetic methods (Ravenhall et al., 2015). The parametric methods look for

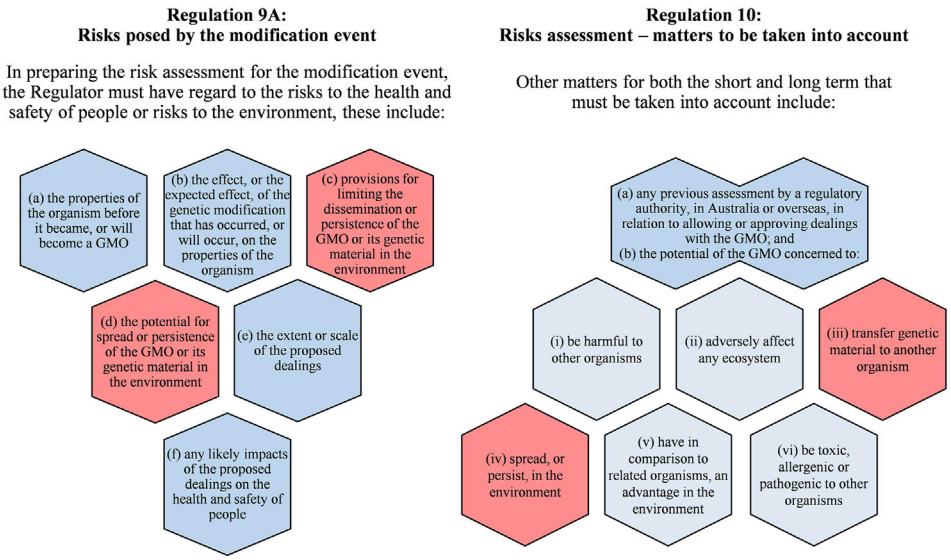


FIGURE 1
Summary of matters prescribed in the Australian Gene Technology Regulations 2001 that the Australian Gene Technology Regulator must consider in the risk assessment for a proposal to release a genetically modified (GM) organism, including a (GM) plant, into the environment (OGTR, 2020). Prescribed matters with a background shading of red may include consideration for horizontal gene transfer.

sections that significantly differ from the average composition; these include GC content and codon usage (Ravenhall et al., 2015). The phylogenetic methods compare the evolutionary histories of a gene of interest and its homologues across multiple species, to identify conflicting phylogenies (Ravenhall et al., 2015; Soucy et al., 2015; Wybouw et al., 2018).

Advances in computational algorithms have also helped in the identification of additional HGT events from genomes that were previously analysed. For example, Wybouw et al. (2018) using refined bioinformatics parameters, identified 25 additional horizontally transferred genes in the spider mite (*Tetranychus urticae*) genome, seven years after HGT was first analysed in this species (Grbic et al., 2011). In the grass species, *Alloteropsis semialata*, initially two genes were identified to be horizontally transferred. Using next generation genome resequencing and strict phylogenetic comparisons amongst 146 other grass species, Dunning et al. (2019) were able to detect 57 additional horizontally transferred genes, some of which are associated with disease resistance and abiotic stress response loci (Dunning et al., 2019). In another example, ToxA, a fungal virulence protein, which is associated with diseases in wheat and barley was shown to reside on a horizontally transferred genomic cluster. The presence of this virulence protein provides a selective advantage to the fungus. Using long-read sequencing technologies, with the available genomes of fungal pathogens in the Pleosporales order, McDonald et al. (2019) were able to confirm the HGT origin of ToxA and define the boundaries of the transferred genomic cluster.

Other examples of HGT events that have been identified between species, using both bioinformatics and experimental methods, include: HGT of genes originating from bacteria, fungi, and plants to bdelloid rotifers (Gladyshev et al., 2008); HGT of carotenoid genes from fungi to pea aphids, causing a red colour polymorphism that provides selective advantage to avoid parasitism compared to green aphids (Losey et al., 1997; Moran and Jarvik, 2010); HGT of a photoreceptor gene from hornworts to ferns, allowing ferns to thrive under low-light conditions (Li et al., 2014); HGT from bacteria and fungi to silkworms of genes thought to confer disease resistance, nutrient and energy metabolism and toxin degradation (Zhu et al., 2011); HGT of an antifreeze protein gene between fish living in icy seawater (Graham et al., 2008), which the authors propose to naturally occur by sperm-mediated HGT during external fertilisation, where the sperm "absorbs" "naked" DNA from the environment; HGT of mitochondrial DNA from the parasite *Trypanosoma cruzi* to humans (Hecht et al., 2010); and HGT from humans to the strictly human pathogen *Neisseria gonorrhoeae* (Anderson and Seifert, 2011). Although far less frequent than HGT between bacteria, HGT from bacteria to eukaryotes has also been described, including the transfer of genes from the bacterium Wolbachia to insects and nematodes (Dunning Hotopp et al., 2007; Nikoh et al., 2008), from bacteria and fungi to plant parasitic nematodes (Noon and Baum, 2016) and from *Agrobacterium* to plants (Matveeva and Otten, 2019). It is to note that the aforementioned HGT examples have non-neutral or advantageous impacts, see section 5 below. A

horizontally transferred gene is unlikely to be maintained in a population if it has a negative impact in the recipient.

Given the automated nature of genome data collection and gene prediction annotation in the contemporary setting, it is impractical to manually validate all genes within a genome. Therefore, concerns relating to whether the inferred HGT events in eukaryotes are statistically supported have been raised (Dupont and Cox, 2017). In addition, the short reads produced by many modern sequencing platforms raises concerns about microorganism contamination, especially involving the putative HGT between these microorganisms (Boothby and Goldstein, 2016; Wickell and Li, 2019). Even though new techniques allow HGT in eukaryotic genomes to be detected with greater frequency than a few years ago, HGT in complex eukaryotes is relatively rare when compared with the observed rates in simpler organisms such as viruses or prokaryotes (Andersson, 2005; Keese, 2008; Vogan and Higgs, 2011; Crisp et al., 2015; Qiu et al., 2016; Sieber et al., 2017).

4 Pathway considerations for HGT from GM plants

Direct or vector-mediated pathways can facilitate HGT. In direct pathways, the recipient organism either "takes up" DNA from another living cell or uptakes "free" or "naked" DNA present in the environment. Vector-mediated pathways are those where DNA is first taken up from the donor by an intermediate recipient that acts as a vector, such as a virus or prokaryote, and then passed on to a different recipient.

There are a number of factors that affect the likelihood of the introduced or modified DNA sequences in GM plants being successfully horizontally transferred and then retained in the final recipient. These include: the proportion of introduced DNA in the GM plant as a source for HGT; the availability and integrity of the introduced DNA sequence; the physical proximity of introduced DNA and a potential recipient organism; whether the recipient organism has a dedicated mechanism for uptake of DNA; whether homologous DNA sequences are present in the recipient organism; whether the donor and recipient are genetically compatible; and whether the horizontally transferred GM DNA sequence gives an advantage to the recipient organism. These factors will be discussed in the following sections.

4.1 Proportion of introduced DNA in GM plants

The likelihood of HGT of the introduced or transgenic DNA from GM plants to other organisms depends on its proportion in relation to the amount of total plant DNA. This proportion can be calculated when both the size of the transgenic insert and the size of the unmodified plant genome are known. As the proportion of the introduced transgenic DNA increases relative to the unmodified genome, so does the likelihood for it to be horizontally transferred.

Crops with single transgenic events, which have been approved for commercial release in Australia, such as altered fatty acid content safflower (GOR-73226-6 and GOR-7324Ø-2) and Roundup Ready™ canola (MON-ØØØ73-7) possess approximately 8.0 kb and 5.05 kb of transgenic DNA in a genome of approximately 2.75 and 1.13 Gb, respectively (Garnatje et al., 2006; Schreiber et al., 2018; Biosafety Clearing-House, 2019). Thus, the transgenic DNA component would account for approximately 0.00029–0.00045% of the total DNA in these crops.

Commercially released crops with stacked transgenic events, such as the six-stacked Agrisure® Duracade™ 5222 corn[1] (SYN-Ø53Ø7-1 × SYN-IR6Ø4-5 × SYN-BTØ11-1 × DAS-Ø15Ø7-1 × MON-ØØØ21-9 × SYN-IR162-4) and four-stacked Bollgard® III × Roundup Ready™ Flex™ cotton (SYN-IR1Ø2-7 × MON-15985-7 × MON-88913-8 × MON 887Ø1-3) possess approximately 40 kb and 30 kb of transgenic DNA, respectively (Biosafety Clearing-House, 2019). With a genome size of corn and cotton at approximately 2.4 Gb (Schreiber et al., 2018), the transgenic DNA would account for approximately 0.0013–0.0017% of total DNA. Therefore, in these stacked event examples, the transgenic DNA occupies a greater proportion of the total crop DNA, and as such an increase in the likelihood of HGT, compared to GM crops with a single transgenic event. That stated, this proportion would be reduced in plants with a larger size genome, such as bread wheat with approximately 17 Gb (Schreiber et al., 2018), than in the previously mentioned stacked transgene examples.

While these GM crops provide examples where the transgenic DNA is introduced at a low copy number into the nuclear genome, other options include introducing transgenic DNA into the mitochondrial or chloroplast genome of plants. Depending on the plant tissue, multiple mitochondria and chloroplast organelles are present within an individual plant cell. Typically, 10 s–100 s of these organelles are present in *Arabidopsis* and tobacco leaf cells (Maliga and Bock, 2011; Sakamoto and Takami, 2018; Shen et al., 2019), with each organelle possessing multiple copies of its genome (Sakamoto and Takami, 2018). For example, if transgenic DNA was introduced into chloroplasts, 100 s–1000 s of copies of the gene are likely to be present per leaf cell (Pontiroli et al., 2010; Sakamoto and Takami, 2018), thereby increasing the proportion of transgenic DNA in the GM plant. Overall, the proportion of transgenic DNA in GM plants, both with single

1 No application has been received for the environmental release of Agrisure® Duracade™ 5222 corn in Australia by the publication date of this manuscript.

and stacked transgenic events, currently authorised for environmental release in Australia represents a minute fraction of the total plant genome.

4.2 Availability and integrity of DNA for HGT

4.2.1 DNA in living plant cells

Plants are frequently exposed to harmful UV radiation, physical shearing and other forces that can damage and alter their DNA. However, DNA in living plant cells is protected through a variety of checking and repair mechanisms. These processes ensure that DNA is maintained to a very high integrity (reviewed in Bray and West, 2005). If the integrity of DNA is not maintained, DNA fragmentation could occur. Should fragmented DNA be horizontally transferred to another organism, it is unlikely to encode a functional protein product. All plant DNA, whether originating in the nucleus, mitochondrion, or chloroplast, is compartmentalised to their respective organelles. In addition to the plant cell wall, this compartmentalisation serves as a physical barrier to limit the availability of DNA to be horizontally transferred from living cells. These physical characteristics would be the same for both GM and non-GM plant DNA.

4.2.2 Naked DNA

When the DNA is no longer contained within cells it is known as "free" or "naked" DNA. Naked DNA is accessible to microorganisms which possess mechanisms to uptake it from their surroundings. Such DNA can arise when: 1) plants deliberately release extracellular DNA, e.g. from their root tips as a defence strategy against soil microbial pathogens (Hawes et al., 2012); and/or 2) after cell death or damage, where the DNA is no longer protected by cell components and is released due to cellular degradation.

The integrity of naked DNA depends on many biotic and abiotic factors (Pontiroli et al., 2007) and most naked DNA is degraded within hours to weeks due to the adverse influence of the surrounding environment. However, small amounts of naked DNA may associate with smaller substrate particle sizes, such as minerals in sand and clay, thereby increasing DNA survival and therefore its availability for HGT (see review by Sand and Jelavić, 2018). It is worth noting that DNA fragments of any size can be internalised by competent prokaryotes and may become incorporated into their genome.

Examples of testing for persistence of naked transgenic DNA are available in the literature. In one experiment the fate of GM transplastomic tobacco DNA and the likelihood of HGT under ideal environmental conditions was investigated. Here the antibiotic resistance gene, *aadA*, which is commonly found in soil bacteria was inserted into the DNA of chloroplasts (Pontiroli et al., 2010). Non-GM and GM tobacco leaf tissue (0.05 g or 0.5 g; whole and ground) and purified GM tobacco DNA were placed into test tubes containing soil and maintained for approximately 4 years. After 4 weeks the amount of total DNA recovered was similar across all samples, however, only 0.002% of total plant DNA was recovered after 4 years. With respect to the transgene, the number of *aadA* gene fragments decreased by more than 10^4-fold over the first 2 weeks, and then by a further 10-fold over the remainder of the experiment. Furthermore, extracted DNA from the soil treatments was transformed into *Acinetobacter* modified to facilitate homologous recombination. Transformed *Acinetobacter* were obtained using total DNA from soil samples containing purified GM tobacco DNA at 0 weeks, but not at later time points. In GM leaf samples, transformants were only obtained using DNA from soil samples that were supplemented with ground 0.5 g of GM leaf discs, but not other leaf treatments.

In another experiment, 2 years after GM sugar beets were harvested, shredded, and disposed, transgenic DNA was detected by PCR in the soil from the disposal site (Gebhard and Smalla, 1999). Although these examples may demonstrate that naked DNA (either intact genes or fragments) can survive for long periods of time, it is currently unknown what this length of time is and the percentage of DNA that would become fragmented. That said, transgenic DNA has the same physical properties as endogenous DNA, resulting in the same likelihood of transgenic DNA being horizontally transferred as that of non-GM plant DNA. A small percentage of naked DNA may therefore be available for HGT not only across time, but also across space as soils and sediments are subject to geological events (noting the degrading effects of abiotic and biotic interactions on DNA integrity).

4.3 Dedicated DNA uptake mechanisms in potential recipients for GM plant DNA

HGT can either occur through a vector-mediated pathway, such as *via* bacteria, viruses, viroids, plasmids or transposons, or *via* a direct pathway, such as exchange and uptake of naked DNA. HGT is most prominent in prokaryotes, especially in bacteria, who utilise it as a mechanism for adaptation, particularly for the acquisition of beneficial traits such as antibiotic resistance when placed under selective pressures (Soucy et al., 2015). HGT in prokaryotes usually occurs *via* conjugation, transformation and transduction. Other mechanisms for HGT include: prokaryotic cell fusion, exchange *via* gene transfer agents, intracellular or endosymbiotic gene transfer, which predominantly pertains to eukaryotes, and introgression. These vast array of mechanisms are thoroughly reviewed in a number of publications, e.g., Soucy et al. (2015) and De Santis et al. (2018).

4.3.1 Conjugation

HGT *via* conjugation requires the physical association between the donor and the recipient cell. A well-characterised

conjugation system occurs between *Agrobacterium* and plants. *Agrobacterium* sp. are soil-based plant pathogens that possess a type IV secretion system (T4SS), allowing the natural transfer and integration of part of its DNA, known as transfer-DNA, or T-DNA to the plant genome (Gelvin, 2017). The presence of historical HGT taking place from naturally occurring *Agrobacterium* has been described in sweet potato (Kyndt et al., 2015), in several *Nicotiana* species (reviewed by Chen and Otten, 2017) and recently in banana and over 30 dicot species, including commonly consumed foods such as peanuts, walnuts, guava, hops (used in beer production) and tea (*Camellia sinensis*, which is used for most teas) (Matveeva and Otten, 2019). In a process known as *Agrobacterium*-mediated transformation, biotechnologists 'disarm' the natural genes on the T-DNA and transform the *Agrobacterium* with a plasmid containing transgenes of interest. As the T4SS can act in *trans*, this modified *Agrobacterium* can be used as a vector to produce GM plants (Gelvin, 2003). However, both biotechnologists and gene technology regulators need to consider genetic elements outside the T-DNA, such as those on the *Agrobacterium* chromosome (Ülker et al., 2008) or on mobile genetic elements (Philips et al., 2017), which in some cases, have also been shown to be horizontally transferred into the plant genome during the *Agrobacterium*-mediated transformation process.

4.3.2 Transformation

Transformation provides another mechanism for HGT, whereby naked DNA is "taken up" from the environment by naturally competent cells, which are predominantly bacteria (Blokesch, 2016). It has been shown under laboratory conditions that approximately 1% of bacterial species can take up DNA from the environment (Mao and Lu, 2016). Transformation can occur in environments where the donor or the intact donor DNA and the receiving organism are in close proximity. With respect to GM plant material, such environments include, but are not limited to, the gastrointestinal tract (GIT) of consumers and the plant phytosphere, which is a complex plant micro-ecosystem comprising of both the exterior and interior of plants that are aboveground and belowground (Yang et al., 2013).

4.3.3 Transduction

Transduction is a process whereby bacteria and archaea acquire DNA *via* HGT, and this process is mediated through phages (Soucy et al., 2015). Transduction can be either generalised or specialised. In generalised transduction, a random piece of the host DNA is incorporated by the phage during lytic phage replication in place of the viral genome. In specialised transduction, an integrated prophage imprecisely excises itself from a host genome and incorporates some of the flanking host DNA (Soucy et al., 2015; Schneider, 2017). These "mistakenly" packaged host DNA can then be horizontally transferred *via* phages to the next bacterium and are likely to occur in environments where phages and bacterium are abundant, such as in waterways and the human GIT (Schneider, 2017).

4.3.4 Gene transfer agents

Gene transfer agents (GTAs) are phage-like particles, found in bacteria and archaea, that can randomly incorporate a piece of the donor's host genome for delivery upon cell lysis to other nearby recipient hosts and as such, can also facilitate HGT (Lang et al., 2012; Soucy et al., 2015). However, GTAs have lost their ability to target their own DNA for packaging. Therefore, they cannot transfer all the genes needed to encode their particle in the new recipient host, creating a distinction from phages participating in transduction (Lang et al., 2012; Soucy et al., 2015).

4.4 Homologous DNA sequences and genetic compatibility

The phylogenetic relationship between the donor and the recipient could also be a major determinant for HGT frequency. Despite the fact that all organisms have a history of HGT (Keese, 2008; Crisp et al., 2015; Fournier et al., 2015), the phylogenetic distance between non-related organisms increases the possibilities of genetic incompatibility, making them less likely to undergo HGT when compared with closely related organisms with compatible genomes (Bertolla and Simonet, 1999; Keese, 2008; Boto, 2010; Hibdige et al., 2021). Conversely, there is a greater likelihood of HGT if homologous regions are present between the donor and recipient (de Vries et al., 2001). Such homologous regions are more likely to be present in closely related organisms, such as between bacteria (Soucy et al., 2015). HGT between bacteria occurs frequently (McAdams et al., 2004). However, based on experimental data, HGT from purified DNA or ground GM plant tissue material to bacteria that lack flanking homologous DNA regions has also been shown to occur. Estimates indicate that this event occurs at a low frequency of 7×10^{-23} per cell. This frequency, as expected, increases if short homologous DNA sequences are present between the donor (GM plant material) and recipient bacteria (7×10^{-13} per cell), but it is still a few orders of magnitude (10^{14}) lower to the naturally occurring rates of HGT between bacteria in the environment (10^{-1} to 10^{-8} per cell) (Dröge et al., 1998; Brigulla and Wackernagel, 2010).

Transgenes which have not originated from plants are generally codon optimised for improved expression when introduced into the GM plant. Prominent examples of bacterial transgenes that have been codon optimised and used in GM plants include the *cry* genes from *Bacillus thuringiensis* imparting insect resistance (Latham et al., 2017) and the *CP4 epsps* gene from *Agrobacterium* sp. strain CP4 imparting resistance to the herbicide glyphosate (Heck et al., 2003). Codon

optimisation can reduce the likelihood of GM plant to bacteria HGT due to the reduction in homology between the optimised transgene and endogenous bacterial sequences. Additionally, if HGT of the intact codon optimised transgene to bacteria were to take place, the encoded protein product would be sub-optimal in expression and may not be retained within the population.

4.5 Proximity of donor DNA to a potential recipient organism

The proximity between the recipient and the donor or the donor's intact DNA is another factor in the likelihood of HGT being successful. Therefore, the relationship between a donor and a symbiont, commensal, epiphyte, pathogen, predator or pest, that facilitate a close physical contact, increase this likelihood (Rumpho et al., 2008; Nikoh and Nakabachi, 2009; de la Casa-Esperón, 2012; Soanes and Richards, 2014; Qiu et al., 2016; Yin et al., 2016; Shinozuka et al., 2017). For plants, the micro-ecosystem comprised by the phytosphere is considered a hotspot for HGT between plants and bacteria (Pontiroli et al., 2009).

Wastewater treatment facilities, where wastewater from a variety of sources, including municipalities, hospitals, and industry converge, are also potential hotspots. This potential is due to the close contact of microorganisms from the variety of different sources, which may form biofilms and the selective pressures caused by pollutants such as heavy metals and antibiotics that can promote HGT (Hultman et al., 2018). The potential for HGT from GM plants to bacteria in wastewater treatment facilities in United States has been described (Gardner et al., 2019).

With respect to GM plants, other hotspots include the GITs of animals and humans after GM plant consumption. For example, the human GIT provides an excellent environment for HGT, with its stable physicochemical conditions and temperature, continuous food supply, high concentration of bacteria and their bacteriophages, and plenty of opportunities for conjugation on the surfaces of food particles and host tissues (Lerner et al., 2017). During the digestive process, consumed food is broken down and fragments of DNA are released in the GIT (see section 4.6.3 for HGT to bacteria in the gastrointestinal tract of humans and animals) and become available for transformation by naturally competent cells. In addition to co-localisation of GM plant DNA and the potential recipient, sufficient time needs to be available for HGT to take place. Bacteria are considered the most likely recipients of HGT from GM plants, because they possess several mechanisms facilitating DNA uptake (see section 4.3 above) and they have many opportunities to form close physical proximity with plants and/or their DNA.

The following sections will describe the likelihood, factors and the barriers for HGT to take place from plants, including GM plants, to a variety of recipients.

4.6 HGT from plants to bacteria

4.6.1 HGT to bacteria in the phytosphere

Despite the fact that potential recipients for transgenic DNA have been identified among soil bacteria (Monier et al., 2007), there is no evidence in the published literature of HGT from a GM plant to soil bacteria under field conditions (Badosa et al., 2004; Demanèche et al., 2008; Ma et al., 2011). For example, the root-associated microbiota was studied in a field 6 years after planting with virus-resistant GM grapevine. In addition to a viral coat protein, the GM grapevine also possessed the *nptII* antibiotic resistance gene (conferring resistance to kanamycin) as a marker. The analysis showed that the presence of GM grapevine did not increase the level of *nptII*-resistant bacteria in the soil, as similar levels of naturally *nptII*-resistant bacteria were found in soil planted with non-GM grapevine (Hily et al., 2018).

4.6.2 HGT to bacteria in aquatic environments

Similar to the considerations for naked DNA in the terrestrial environment, naked DNA in the aquatic setting also needs to remain intact for the likelihood of aquatic microorganisms to incorporate this DNA into their genome and then produce its functional protein product. The persistence of naked DNA in water samples (groundwater and river water) originating from GM corn (event MON-ØØ863-5) and purified plasmid DNA, both containing the *nptII* antibiotic resistance gene, was measured by the ability of *Pseudomonas stutzeri* to naturally take up the naked DNA (Zhu, 2006). The results, based on *P. stutzeri's* natural uptake, showed the presence of the plasmid DNA in intact or filter sterilised water but that this decreased to undetectable levels within 4 days (Zhu, 2006), indicating that elements in these water samples aided DNA degradation. Likewise, in the same study, the stability of GM plant DNA was assessed by real-time PCR. The results demonstrated that the concentration of GM plant DNA reduced by two orders of magnitude within 4 days in intact and filter sterilised water (Zhu, 2006). Thus, material such as pollen, leaves, fruit and other plant detritus, originating from GM plants could potentially make its way to the aquatic environment and become available for HGT should its DNA maintain integrity (Poté et al., 2009).

4.6.3 HGT to bacteria in the gastrointestinal tract of humans and animals

In their diets, humans and animals are regularly exposed to DNA from a variety of sources, including from plants, animals and microorganisms. Nawaz et al. (2019) reviewed that as part of a normal human diet, the daily dietary intake of DNA ranged between 0.1 and 1 g. The likelihood of HGT of transgenic DNA from GM plants to gut bacteria or tissues of animals and humans is very low when considering the total pool of all available DNA in the GIT (Jennings et al., 2003; Netherwood et al., 2004; Sieradzki et al., 2013; Korwin-Kossakowska et al., 2016).

Estimates for the percentage of GM DNA in a theoretical Austrian daily diet were performed by Jonas et al. (2001). As part of their daily average diet, Austrians would consume 170 g of soybean, maize, and potato. Based on a total daily dietary DNA intake of 0.6 g, and considering the consumption of purely GM crops, approximately 0.00006% of the total DNA would be GM (Jonas et al., 2001). Similarly, in dairy cows consuming 60% GM maize, approximately 0.000094% of the total daily DNA intake would be GM (Beever and Phipps, 2001). However, these estimated percentages are based on intact DNA prior to consumption and have not considered the fate of the DNA during the digestive process. As the DNA from GM organisms, including GM animals, insects and plants is chemically equivalent to DNA from other sources, the fate of GM DNA in the GIT is similar to that of non-GM DNA (Rossi et al., 2005; Van Eenennaam and Young, 2014). This fate is purely for the DNA and there would be separate considerations for regulators for any consumed proteins encoded by the GM DNA, which is beyond the scope of this review.

There are several factors that detrimentally affect the integrity and availability of DNA. These include the process of food preparation, cooking, and digestion of DNA in the GIT, all of which fragment the DNA (these factors have been reviewed in Rizzi et al. (2012) and Nawaz et al. (2019)). Thus, only in rare circumstances is it likely that an intact gene or a transgene is able to participate in HGT from dietary sources to consumers or to the bacteria in their GIT.

In insects, the discovery of incompletely digested leaf fragments in the faeces of tobacco hornworm fed on GM transplastomic tobacco carrying the *nptII* gene raised the possibility that gut bacteria could uptake GM plant DNA (Deni et al., 2005). However, this could not be confirmed in the gut bacteria of the species tested so far, which include tobacco hornworm and bees (Deni et al., 2005; Mohr and Tebbe, 2007; Hendriksma et al., 2013; Niu et al., 2017). Experiments to investigate HGT to the bacteria in the GIT of birds and mammals have also been undertaken. For example, GM corn, GM rice, GM soybean or purified plasmid DNA were introduced in the diets of rats, broilers, laying hens, pigs, piglets and calves. No instances of HGT of the introduced DNA to bacteria in the GIT was observed in these experiments (Nemeth et al., 2004; Zhu et al., 2004; Deaville and Maddison, 2005; Calsamiglia et al., 2007; Wilcks and Jacobsen, 2010; Yonemochi et al., 2010; Walsh et al., 2011; Nordgård et al., 2012; Sieradzki et al., 2013; Świątkiewicz et al., 2013; Zhao et al., 2016).

Overall, HGT from GM plants to bacteria has rarely been reported (Nielsen et al., 1998; Andersson, 2005; Pontiroli et al., 2009; Miyashita et al., 2013). This is likely to be a consequence of the small percentage of introduced DNA in GM plants (see section 4.1 above) combined with the low HGT frequency from plants to prokaryotic recipients (Pontiroli et al., 2009; Brigulla and Wackernagel, 2010).

4.7 HGT from plants to eukaryotes

4.7.1 Direct HGT to humans and animals

Animals are multicellular eukaryotes whose cells lack walls. Most animals cannot synthesise their own nutrients, but instead rely on obtaining these by digesting other organisms as food. If plant material is consumed, its DNA will be present in the animal's GIT (Broaders et al., 2013). For HGT to become a reality, the consumed DNA would have to maintain its integrity after digestion, be horizontally transferred to the reproductive cells and then be passed on to the recipient's offspring. This could be achieved by consumed DNA being transferred into germline cells either directly, if these are physically close to the digestive system, or *via* a circulation system, such as the blood in vertebrates, or the haemolymph in lower animals.

The presence of GM DNA in a variety of higher animals who have consumed GM plants as part of their diet have been tested. For example, herbicide tolerant Roundup Ready® soybean (event MON-Ø4Ø32-6) and insect resistant corn (event MON-ØØ81Ø-6) were used in feeding experiments that were carried out over ten generations on Japanese quails. The results showed no signs of GM DNA in tissue samples, including the breast muscle, eggs and internal organs (Korwin-Kossakowska et al., 2016). In other studies, fragments of ingested DNA have been detected in the blood of humans and a variety of higher animals, which have been extensively reviewed (e.g., Parrott et al., 2010; Nicolia et al., 2014; Nadal et al., 2018). Small amounts of fragmented DNA have also been shown to be absorbed into the gut epithelial tissues of mammals (Rizzi et al., 2012). It is to note that fragmented DNA may no longer be able to encode a protein in its entirety and as such is unlikely to be functionally active. In other reports, small fragments of GM DNA have been detected in some tissue samples from pigs, sheep and birds (Jennings et al., 2003; Mazza et al., 2005; Rossi et al., 2005; Sharma et al., 2006). Albeit present in some tissues, there was no evidence of GM DNA integration into the genome of somatic cells, or its transfer into the germ cell DNA in these animals.

In addition, there has been no GM DNA or protein detected in consumed products such as milk, meat or eggs from livestock that have been with fed GM plants (reviewed by Van Eenennaam and Young, 2014; De Santis et al., 2018). Nawaz et al. (2019) suggest that uptake of fragmented DNA into the bloodstream of consumers is a common occurrence. Testing in these studies was generally conducted within 24 h after consumption and detection of the ingested DNA was most likely to originate from high-copy-number genes, such as those present in the chloroplast (reviewed in Nadal et al., 2018; Nawaz et al., 2019). However, after 24 h, the ingested DNA present in the blood was difficult to detect, indicating that there are mechanisms in place to eliminate them (Nawaz et al., 2019).

An important consideration for multicellular eukaryotes is that the horizontally acquired genes would need to reach the germ line and then be transferred to the next generation. This

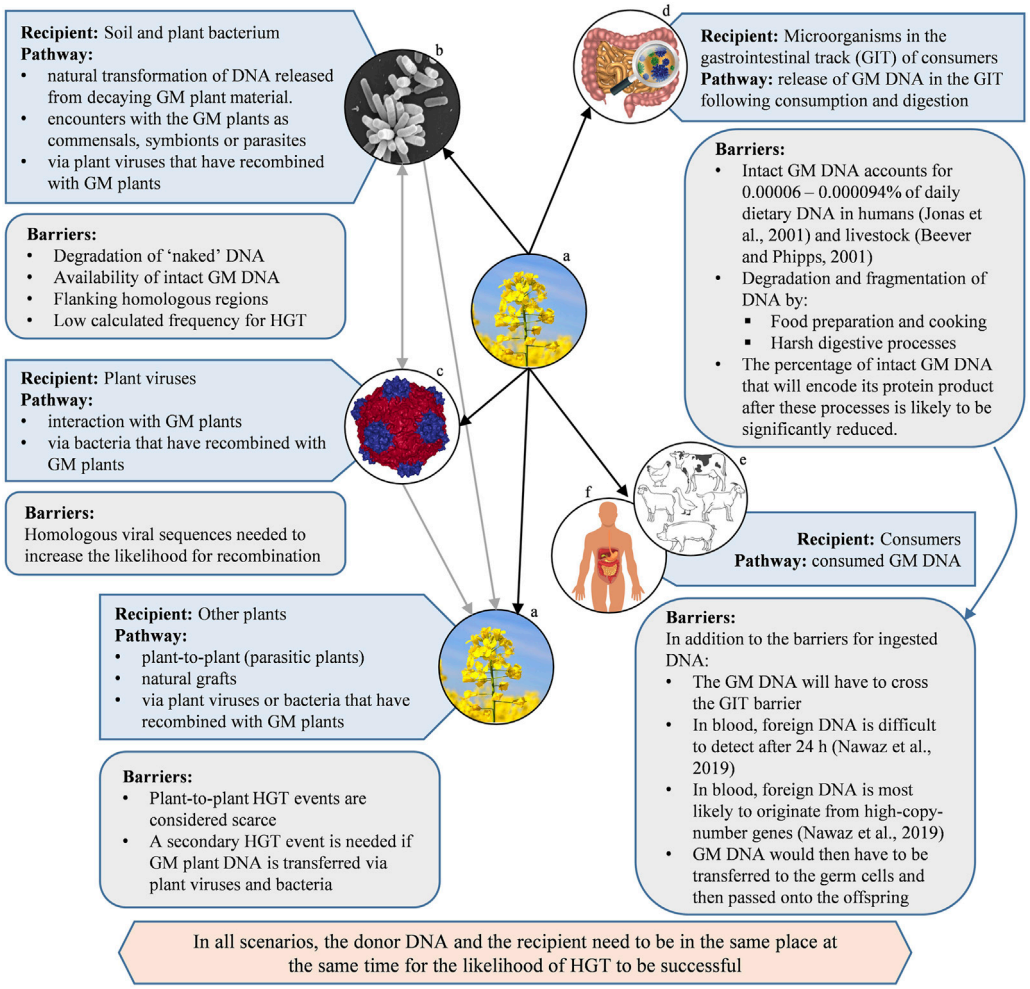


FIGURE 2
A selection of pathways and barriers for the horizontal gene transfer (HGT) from (GM) plants to a selection of recipients. Black arrows indicate direct HGT, grey arrows indicate a secondary HGT event from plant viruses and/or bacterium. [Images courtesy of: **(A)** canola by Pixabay/Jenő Szabó, **(B)** *Agrobacterium* by Jing Xu, Indiana University, **(C)** Cowpea mosaic virus, PDB ID: 1NY7 (Lin et al., 1999), created using NGL viewer (Rose et al., 2018) at RCSB PDB, **(D)**, **(E)** and **(F)** sourced from iStock].

entails an extra barrier for HGT and chances of transmission of horizontally transferred genes to offspring are rare, even if transmission happens during unicellular or early developmental stages (Huang, 2013). In an alternative pathway, a fetus may be exposed to DNA fragments when the pregnant mother consumes DNA-containing material. In studies conducted in the late 1990s, pregnant mice were fed with high levels of purified phage M13 and plasmid DNA. The presence of this foreign DNA was then tested in the fetuses and in new-born mice, where fragmented phage M13 DNA was detected (Schubbert et al., 1997; Schubbert et al., 1998). The results showed that not all cells in the fetuses or new-born mice contained this foreign DNA. It was concluded that the DNA fragments were most likely transferred across the placenta from the mother. It was not clear if DNA fragments integrated into the genome of the somatic cells of the offspring or if they were present transiently. The limitations and conclusions of this study have been extensively critiqued by Beever and Kemp (2000). A follow-up study by the original researchers found no transfer to the germline cells when mice were fed transgenic DNA daily over eight generations (Hohlweg and Doerfler, 2001).

In summary, if an animal diet includes the consumption of GM plants, there are several barriers that need to be overcome for HGT of the transgene to take place (Figure 2). These include: The extremely low percentage of the GM DNA in the overall dietary intake, which is chemically equivalent to non-GM DNA; the fragmentation of DNA due to digestion, reducing the likelihood of the GM DNA to code for a functional product; the additional hurdle of the GM DNA crossing the gastrointestinal barrier; and persisting in the bloodstream for it to be available for

incorporation into germline cells. If all these challenges could be overcome, and for HGT to be successful, the GM DNA would then have to be passed onto the consumer's offspring.

4.7.2 HGT between plants

Plants are multicellular eukaryotes, most of which are capable of synthesising their own nutrients. Plant cells possess a cell wall that is made up of cellulose, which adds a physical barrier in accessing the DNA within living cells or taking up DNA from other plants. The intimate contact required for HGT between plants may occur in natural or artificial grafts, or *via* parasitic interactions. During these interactions, an exchange of substances, including nucleic acids can occur. Although epiphytic plants are in contact with their host throughout their lives, there is only superficial contact with the surface of the host rather than tight interaction between the two. This is a barrier to direct HGT between epiphytes and their hosts.

4.7.2.1 Direct HGT of nuclear plant DNA to other plants

The strongest evidence of plant-to-plant HGT occurring are those between parasitic plants and their hosts (Xi et al., 2013; Davis and Xi, 2015; Yang et al., 2016; Yoshida et al., 2016; Yang et al., 2019). This is most likely due to formation of a multicellular organ called the haustorium when the parasitic plant encounters the host. The haustorium creates an intimate physical association by penetrating into the host stem or root and then connecting to the host vasculature, which allows the exchange of a wide range of materials including DNA and RNA (Yoshida et al., 2016).

Some recent studies of HGT involving plants, predominantly in grasses, have been described. Such examples include: multiple HGTs of nuclear ribosomal genes between grass lineages (Mahelka et al., 2017); HGT between distantly related grasses of a second enzymatic gene that aids in microhabitat variation (Prentice et al., 2015); and evidence of the contribution of nuclear HGT to C_4 evolution in grasses (Christin et al., 2012). More recently, genomes of a diverse set of 17 grass species that span more than 50 million years of divergence were analysed for grass-to-grass protein-coding HGT events. The results indicated that major crops, such as maize and wheat were recipients to horizontally transferred genes (Hibdige et al., 2021).

4.7.2.2 Direct HGT of non-nuclear plant DNA to other plants

In general, HGT of non-nuclear DNA, i.e., mitochondrial and chloroplast DNA, between individual plants is considered to be more likely than nuclear DNA transfer due a variety of factors including; their high copy number and a process known as organelle capture (Stegemann et al., 2012). For example, in natural grafts, where two plant stems or roots are in contact with each other, or under laboratory grafting experiments, the transfer of entire chloroplast genomes or even full mitochondria organelles have been detected (Stegemann and Bock, 2009; Stegemann et al., 2012; Thyssen et al., 2012; Gurdon et al., 2016). However, some authors suggest that heritable changes might only be possible if the formation of lateral shoots occurs within the graft site (Stegemann and Bock, 2009), certainly heritable changes can be induced following grafting under laboratory conditions (Fuentes et al., 2014). As such, the stability of horizontally transferred genes *via* natural grafting (regarding integration, expression, and inheritability) requires additional analysis (Gao et al., 2014).

Transfer of non-nuclear DNA has also been shown to occur independently of grafting. For example, the whole genome analysis of *Amborella trichopoda*, which is thought to be the most basal extant flowering plant revealed six genome equivalents of historical horizontally acquired mitochondrial DNA. These were acquired from green algae, mosses, and other angiosperms and some transferred as intact mitochondria (Rice et al., 2013). Non-nuclear DNA transfer also occurs in parasitic interactions (Xi et al., 2013; Davis and Xi, 2015; Yoshida et al., 2016; Sanchez-Puerta et al., 2019; Sinn and Barrett, 2019) and mitochondrial HGTs in both directions have been detected in 10 of 12 parasitic lineages (Yoshida et al., 2016).

The number of chloroplasts per plant cell is highly variable, with approximately 100–120 chloroplasts per cell in the leaves of tobacco and *Arabidopsis* (Maliga and Bock, 2011). Thus, GM plants possessing the transgene in the chloroplast, for example, have a much higher transgene copy-number than nuclear-modified plants. In addition, as the chloroplast is prokaryotic in origin, it is more likely to share homologous regions with other prokaryotes. Therefore, transgenes within the chloroplasts of GM plants have been proposed to increase the likelihood of HGT to bacteria compared to transgenes integrated into nuclear DNA (Kay et al., 2002; Monier et al., 2007). However, studies comparing plasmid DNA, PCR products and chloroplast-transformed tobacco, containing ~7,000 copies of the transgene per plant cell, all of which contained the same DNA construct concluded that there was no indication that these high-copy-number chloroplast transformed GM plants could cause higher rates of HGT than nuclear-transformed GM plants (Demanèche et al., 2011).

Currently, to the best of our knowledge, there have been no reports of HGT from a GM to a non-GM plant.

4.8 HGT from plants to viruses

Plant viruses could also be recipients of genes horizontally transferred from GM plants. Viruses frequently evolve by recombination between homologous viral sequences (Keese, 2008). Therefore, GM plants carrying virus-derived sequences, such as viral promoters, might be more likely to act as an HGT donor for plant viruses capable of infecting these GM plants (Keese, 2008).

An *in vivo* study of HGT from GM grapevine was carried out by assessing its root-associated microbiota 6 years after planting (Hily

et al., 2018). The grapevine was modified by introducing the coat protein from *Grapevine fanleaf virus* (GFLV) strain F13 (*F13-cp*) to confer resistance against the virus as well as the *nptII* marker gene as previously discussed. For the viral transgene, analysis of the GFLV population showed a large number of natural recombination events within the virus; however, none of these recombinants contained the *F13-cp* or *nptII* transgene sequence (Hily et al., 2018).

Under laboratory conditions, plant viruses demonstrate the ability to incorporate plant DNA or RNA into their genome. For example, experiments were conducted with *Cucumber necrosis virus* (CNV), which is a positive-sense, single-stranded RNA virus. When *Nicotiana benthamiana* leaves were infiltrated with the transcript of CNV coat protein, virus-like particles were produced that carried a variety of host RNAs, including retrotransposons and chloroplast-specific RNAs (Ghoshal et al., 2015). In the case of retrotransposons, the authors concluded that it would be possible for these to be horizontally transferred *via* the virus to new hosts (Ghoshal et al., 2015). Likewise, the *Beet curly top Iran virus* (BCTIV), a single-stranded DNA virus, can incorporate DNA from its sugar beet host to form hybrid virus-plant minicircles. These can then be packaged and have been shown to replicate and be transcribed in other plant species sensitive to BCTIV infection (Catoni et al., 2018).

4.9 HGT from plants to other organisms and facilitation *via* vectors

The introduced DNA in GM plants has the potential to be horizontally transferred to other classes of organisms that have not been mentioned in this review, either through a direct or a vector-mediated pathway. Such organisms include, but are not limited to, algae, fungi, or nematodes. For example, rare HGT events from plants to fungi have been reported (Richards et al., 2009; Nikolaidis et al., 2013; Li et al., 2018). Should HGT from plants to recipients, such as fungi and bacteria take place, the possibility of the recipient itself acting as a secondary HGT donor/vector becomes available.

Other potential recipients include arthropods and nematodes, which also have a history of horizontally acquiring genes from bacteria and fungi (Mitreva et al., 2009; Haegeman et al., 2011; Wybouw et al., 2016). Similarly, viruses could act as a HGT vector facilitating gene transfer from plants to bacteria. However, viruses that function in both plants and bacteria are rare (Nielsen et al., 1998), although certain plant viruses, such as geminiviruses, have been shown under experimental conditions to replicate in the bacterium, *Agrobacterium tumefaciens* (Rigden et al., 1996). If HGT was to then take place between the virus and bacterium under field conditions, a secondary vector-mediated pathway could become available, with bacteria then acting as a HGT donor to other plants. Thus, in this scenario, HGT *via* two vectors could take place between GM and non-GM plants.

Likewise, bacteria could horizontally transfer DNA that it has acquired from GM plants *via* HGT to other organisms. However, these successive processes would most likely require several more barriers to be overcome, each reducing the likelihood for successful HGT, and would most likely need to be carried out over an evolutionary timescale.

5 Considerations regarding the potential for adverse outcomes as a consequence of HGT

As previously discussed, while the acquisition of a new gene by HGT is not considered harm per se, it has the potential to lead to genetic variation within a population, and thus, its impact on driving the evolutionary function of organisms has been considered (Keese, 2008; Boto, 2010). With respect to GM plants, for HGT-induced harm, the acquisition of the genetic material must result in a non-neutral change for the recipient, be maintained in the population and result in an adverse outcome to humans, animals and/or the environment (Keese, 2008).

Occasionally, the function of the transferred genes could strongly affect the severity of the adverse consequences or the likelihood of a HGT event (Keese, 2008). When a population is under strong selective pressures or environmental stresses, HGT can be stimulated. In the recipient, the transferred gene can either confer a detrimental, neutral, or advantageous trait. If this novel trait is advantageous, the recipient can overcome its pressures and stresses, outcompete its neighbours or adapt to a new ecological niche (van Elsas et al., 2003; Keeling, 2009; Raz and Tannenbaum, 2010; Vogan and Higgs, 2011; de la Casa-Esperón, 2012). For example, under intensive agricultural production, the coffee berry borer beetle, *Hypothenemus hampei*, horizontally acquired a mannanase gene from bacteria that helped it adapt to enzymatically digest the polysaccharides of coffee beans, converting it into an invasive pest (Acuña et al., 2012). Similarly, the whitefly, *Bemisia tabaci*, horizontally acquired a phenolic glucoside malonyltransferase gene from plants allowing it to neutralise the plant-produced phenolic glycosides that would otherwise kill the whitefly after herbivory. This to our knowledge is the first known example of a HGT event between a plant and an animal and is thought to have occurred ~86 million years ago (Xia et al., 2021). There are also instances that illustrate how novel genes acquired by HGT contributed to parasite adaptation to a new host in different organisms, for example: HGT of cellulase genes, allowing cellulase activity, from several microbial donors to nematodes, which enhances their parasitism and pathogenicity of plants (Danchin et al., 2010); HGT of genes associated with disease resistance and abiotic stress response in spider mites (Grbic et al., 2011; Wybouw et al., 2018); and HGT of fungal genes involved in the metabolism of plant sugars and genes involved in the breaking down of cell walls to increase the parasitism of oomycetes (Richards et al., 2011).

Even when an organism horizontally acquires a gene that leads to a selective advantage, the advantage might not have a

short-term impact on the recipient's ecology, and changes might only be significant when considered in an evolutionary timescale (Hiltunen et al., 2017). Similarly, the ecological benefits of an adaptation acquired by a sporadic HGT event could dissipate over time (Hiltunen et al., 2017). In these cases, the impacts to the ecosystem of a casual HGT event are difficult to assess, and risk assessments cannot rely on considering just HGT frequency, as this is not a good prognostic tool for long term effects of HGT (Pettersen et al., 2005).

Many adverse effects owing to the potential of HGT of the transgene from GM plants to other organisms, including humans, are gene dependent. These effects include their potential role in allergenicity, pathogenicity, virulence, toxicity and other environmental effects. Therefore, these potential effects should be evaluated on a case-by-case basis in the context of the proposed activities in the risk assessment of each GM plant if occurrence of HGT is considered more likely than when dealing with the non-GM plant.

For example, one of the most common concerns regarding GM plant safety is the potential environmental and health consequences of HGT with regards to prokaryotic-derived antibiotic resistance genes, which are predominantly used as markers to select for the transformation event. Should HGT and subsequent integration occur to the gut microflora of consumers, including humans, the concern relates to the proliferation of antibiotic resistant strains of harmful bacteria which would be harder to control (Rizzi et al., 2012). These concerns have been previously assessed (see EFSA (2017) and references within Woegerbauer et al. (2015)) and there is no account of such an event occurring from a GM plant source under field conditions (Demanèche et al., 2008; Pilate et al., 2016; EFSA et al., 2017; Tsatsakis et al., 2017; Hily et al., 2018). In addition, the presence of these resistance genes has not significantly increased antibiotic resistance in the clinical setting (Breyer et al., 2014). Furthermore, natural antibiotic and herbicide resistance genes are found in widely dispersed soil microorganisms, often on mobile genetic elements (Pontiroli et al., 2007) and the HGT of these naturally occurring genes has previously been described by Domingues et al. (2012). Therefore, these naturally occurring microorganisms, as well as the vast pool of available antibiotic resistance genes already naturally present in the intestinal microflora in the human GIT, are far more likely to be the HGT source for these resistance genes than GM plants (Pontiroli et al., 2007; Tothova et al., 2010; Huddleston, 2014).

That stated, the evaluation of antibiotic resistance genes in GM plants needs to be considered on a case-by-case basis and regulators at the European Food Safety Authority (EFSA) have adopted the classification of these genes into three risk groups. These groups class antibiotic resistance genes based on their abundance in the environment and their significance to human and veterinary medicine, reviewed in De Santis et al. (2018). Regulators in Europe have also issued a directive to phase out antibiotic resistance genes used in GM plants that have adverse effects on human health and the environment [Directive 2001/18/EC of the European Parliament and of the Council (on the deliberate release into the environment of genetically modified organisms)] (Garcia, 2006). As such, biotechnologists are encouraged to develop GM plants without the use of antibiotic resistance gene markers (Breyer et al., 2014). In Australia, the risk assessment process considers the background presence of the gene(s) used in the genetic modification "Antibiotic resistance marker genes commonly used in the selection process for generating GM plants are derived from soil bacteria abundant in the environment. Therefore, exposure to an antibiotic resistance gene, or to the protein encoded by such a gene, derived from a GMO, may or may not be significant against the naturally occurring background" (OGTR, 2013).

6 Conclusion

HGT is most prominent in prokaryotes, as many lack sexual recombination and thus employ HGT as a mechanism for adaptation to the environment. For example, the beneficial acquisition of antibiotic resistance through HGT in a clinical setting, and several other cases of HGT have been researched and documented in this context. Despite the significant role that HGT has played in the evolution of eukaryotic genomes, nuclear HGT events between multicellular eukaryotes are considered scarce, when compared to those between prokaryotes and in either direction between prokaryotes and eukaryotes (Richardson and Palmer, 2007; Keeling and Palmer, 2008; Keeling, 2009; Bock, 2010; Huang, 2013; Gao et al., 2014; Schönknecht et al., 2014). Specifically, scarcity of HGT between plants can be attributed to the need of a vector to facilitate the transfer (Mahelka et al., 2017).

Overall, the frequency of HGT for all organisms, including viruses and bacteria, is orders of magnitude lower than gene transfer by sexual or asexual reproduction. This is due to HGT needing to overcome numerous barriers, such as those related to the transfer, incorporation, and transmission of the DNA between organisms. In eukaryotes, additional barriers are needed to be overcome, where the DNA may first need to be transferred from the somatic to germ cell line and then be transferred to the recipient's offspring.

The advances in whole genome sequencing and comparative genomics demonstrates that, although historical, HGT events in eukaryotic organisms may have previously been underestimated. (Bock, 2010; Crisp et al., 2015; Drezen et al., 2017; Quispe-Huamanquispe et al., 2017; Sieber et al., 2017; Matveeva and Otten, 2019). However, by the publication date of this manuscript, there have been no reports of adverse impacts on human health or environmental safety as a direct or indirect result of HGT from GM plants. Moreover, in the Australian context, GM plants approved for environmental release often contain genes and regulatory sequences that originate from naturally occurring organisms that are already present in the environment. Therefore, the potential for unintended adverse effects of HGT of the inserted genetic material is unlikely to be

greater than those by its naturally occurring genetic counterparts. As such, the potential for adverse effects to human and animal health and to the environment as a result of HGT from GM plants already authorised for environmental release in Australia are highly unlikely. However, it is worth considering that, by exchanging the host of the introduced genetic material, a closer physical association to a potential recipient might be enabled, potentially increasing the likelihood for HGT. Furthermore, with recent advances in genome editing, non-food plants, such as *N. benthamiana*, are likely to be genetically modified with DNA sequences encoding components for production of pharmaceuticals and vaccines (Bally et al., 2018). The DNA sequences in such GM plants may be novel or synthetic, and therefore are unlikely to already be present in the environment. This would pose new challenges to gene technology regulators in conducting their risk analysis, as a direct comparator is not immediately apparent. The Australian approach provided by the Regulator's Risk Analysis Framework (OGTR, 2013) would still allow the risk assessment of any novel or synthetic DNA sequence in the GM plant to be conducted. Similarly, the risks associated from gene-edited plants would also follow the above processes, noting that in Australia, organisms modified *via* Site Directed Nucleases (SDN) without guide RNAs (SDN-1) are organisms that are not genetically modified organisms under Schedule 1 of the Gene Technology Regulations 2001 for regulatory purposes following the changes made to the Australian Regulations in 2019 (OGTR, 2020; O'Sullivan et al., 2022).

Author contributions

JP wrote, revised, edited, coordinated and submitted the manuscript. EM-A conceived the manuscript, wrote the initial draft and with AR critically revised and edited the manuscript.

Acknowledgments

The authors would like to thank Alison Wardrop, Kylie Tattersall, Heidi Mitchell and other staff at the Office of the Gene Technology Regulator for useful discussions and helpful comments on a draft of this manuscript. We also thank the editor and the reviewers for their valuable comments on the manuscript.

Author disclaimer

All claims expressed in this article are solely those of the authors and do not necessarily represent those of the Australian Gene Technology Regulator or the Office of the Gene Technology Regulator.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

ACRE (Advisory Committee on Releases to the Environment) (2013). *Report 1: Towards an Evidence-Based Regulatory System for GMOs*. London.

Acuña, R., Padilla, B. E., Flórez-Ramos, C. P., Rubio, J. D., Herrera, J. C., Benavides, P., et al. (2012). Adaptive Horizontal Transfer of a Bacterial Gene to an Invasive Insect Pest of Coffee. *Proc. Natl. Acad. Sci. U. S. A.* 109 (11), 4197–4202. doi:10.1073/pnas.1121190109

Anderson, M. T., and Seifert, H. S. (2011). Opportunity and Means: Horizontal Gene Transfer from the Human Host to a Bacterial Pathogen. *mBio* 2 (1), e00005–00011. doi:10.1128/mBio.00005-11

Andersson, J. O. (2005). Lateral Gene Transfer in Eukaryotes. *CMLS. Cell. Mol. Life Sci.* 62 (11), 1182–1197. doi:10.1007/s00018-005-4539-z

Badosa, E., Moreno, C., and Montesinos, E. (2004). Lack of Detection of Ampicillin Resistance Gene Transfer from Bt176 Transgenic Corn to Culturable Bacteria under Field Conditions. *FEMS Microbiol. Ecol.* 48 (2), 169–178. doi:10.1016/j.femsec.2004.01.005

Bally, J., Jung, H., Mortimer, C., Naim, F., Philips, J. G., Hellens, R., et al. (2018). The Rise and Rise of *Nicotiana Benthamiana*: A Plant for All Reasons. *Annu. Rev. Phytopathol.* 56 (1), 405–426. doi:10.1146/annurev-phyto-080417-050141

Beever, D. E., and Kemp, C. F. (2000). Safety Issues Associated with the DNA in Animal Feed Derived from Genetically Modified Crops. A Review of Scientific and Regulatory Procedures. *Nutr. Abstr. Rev. Ser. B, Livest. Feed Feed.* 70 (3), 197–204.

Beever, D. E., and Phipps, R. H. (2001). The Fate of Plant DNA and Novel Proteins in Feeds for Farm Livestock: A United Kingdom Perspective. *J. Animal Sci.* 79, E290–E295. doi:10.2527/jas2001.79E-SupplE290x

Bertolla, F., and Simonet, P. (1999). Horizontal Gene Transfers in the Environment: Natural Transformation as a Putative Process for Gene Transfers between Transgenic Plants and Microorganisms. *Res. Microbiol.* 150 (6), 375–384. doi:10.1016/S0923-2508(99)80072-2

Biosafety Clearing-House (2019). Living Modified Organism (LMO) Registry. Available: http://bch.cbd.int/database/lmo-registry/(Accessed 19 11, 2019).

Blokesch, M. (2016). Natural Competence for Transformation. *Curr. Biol.* 26 (21), R1126–R1130. doi:10.1016/j.cub.2016.08.058

Bock, R. (2010). The Give-And-Take of DNA: Horizontal Gene Transfer in Plants. *Trends Plant Sci.* 15 (1), 11–22. doi:10.1016/j.tplants.2009.10.001

Boothby, T. C., and Goldstein, B. (2016). Reply to Bemm et al. and Arakawa: Identifying foreign genes in independent *Hypsibius dujardini* genome assemblies. *Proc. Natl. Acad. Sci. U. S. A.* 113 (22), E3058–E3061. doi:10.1073/pnas.1601149113

Boto, L. (2010). Horizontal Gene Transfer in Evolution: Facts and Challenges. *Proc. R. Soc. B* 277 (1683), 819–827. doi:10.1098/rspb.2009.1679

Bray, C. M., and West, C. E. (2005). DNA Repair Mechanisms in Plants: Crucial Sensors and Effectors for the Maintenance of Genome Integrity. *New Phytol.* 168 (3), 511–528. doi:10.1111/j.1469-8137.2005.01548.x

Breyer, D., Kopertekh, L., and Reheul, D. (2014). Alternatives to Antibiotic Resistance Marker Genes for *In Vitro* Selection of Genetically Modified Plants – Scientific Developments, Current Use, Operational Access and Biosafety Considerations. *Crit. Rev. Plant Sci.* 33 (4), 286–330. doi:10.1080/07352689.2013.870422

Brigulla, M., and Wackernagel, W. (2010). Molecular Aspects of Gene Transfer and Foreign DNA Acquisition in Prokaryotes with Regard to Safety Issues. *Appl. Microbiol. Biotechnol.* 86 (4), 1027–1041. doi:10.1007/s00253-010-2489-3

Broaders, E., Gahan, C. G., and Marchesi, J. R. (2013). Mobile Genetic Elements of the Human Gastrointestinal Tract: Potential for Spread of Antibiotic Resistance Genes. *Gut Microbes* 4 (4), 271–280. doi:10.4161/gmic.24627

Calsamiglia, S., Hernandez, B., Hartnell, G. F., and Phipps, R. (2007). Effects of Corn Silage Derived from a Genetically Modified Variety Containing Two Transgenes on Feed Intake, Milk Production, and Composition, and the Absence of Detectable Transgenic Deoxyribonucleic Acid in Milk in Holstein Dairy Cows. *J. Dairy Sci.* 90 (10), 4718–4723. doi:10.3168/jds.2007-0286

Catoni, M., Noris, E., Vaira, A. M., Jonesman, T., Matić, S., Soleimani, R., et al. (2018). Virus-mediated Export of Chromosomal DNA in Plants. *Nat. Commun.* 9 (1), 5308. doi:10.1038/s41467-018-07775-w

Chen, K., and Otten, L. (2017). Natural *Agrobacterium* Transformants: Recent Results and Some Theoretical Considerations. *Front. Plant Sci.* 8 (1600). doi:10.3389/fpls.2017.01600

Christin, P.-A., Edwards, Erika J., Besnard, G., Boxall, Susanna F., Gregory, R., Kellogg, Elizabeth A., et al. (2012). Adaptive Evolution of C_4 Photosynthesis through Recurrent Lateral Gene Transfer. *Curr. Biol.* 22 (5), 445–449. doi:10.1016/j.cub.2012.01.054

Crisp, A., Boschetti, C., Perry, M., Tunnacliffe, A., and Micklem, G. (2015). Expression of Multiple Horizontally Acquired Genes Is a Hallmark of Both Vertebrate and Invertebrate Genomes. *Genome Biol.* 16 (1), 50. doi:10.1186/s13059-015-0607-3

Danchin, E. G. J., Rosso, M.-N., Vieira, P., de Almeida-Engler, J., Coutinho, P. M., Henrissat, B., et al. (2010). Multiple Lateral Gene Transfers and Duplications Have Promoted Plant Parasitism Ability in Nematodes. *Proc. Natl. Acad. Sci. U. S. A.* 107 (41), 17651–17656. doi:10.1073/pnas.1008486107

Davis, C. C., and Xi, Z. (2015). Horizontal Gene Transfer in Parasitic Plants. *Curr. Opin. Plant Biol.* 26, 14–19. doi:10.1016/j.pbi.2015.05.008

de la Casa-Esperón, E. (2012). Horizontal Transfer and the Evolution of Host-Pathogen Interactions. *Int. J. Evol. Biol.* 9, 1. doi:10.1155/2012/679045

De Santis, B., Stockhofe, N., Wal, J.-M., Weesendorp, E., Lallès, J.-P., van Dijk, J., et al. (2018). Case Studies on Genetically Modified Organisms (GMOs): Potential Risk Scenarios and Associated Health Indicators. *Food Chem. Toxicol.* 117, 36–65. doi:10.1016/j.fct.2017.08.033

de Vries, J., Meier, P., and Wackernagel, W. (2001). The Natural Transformation of the Soil Bacteria *Pseudomonas Stutzeri* and *Acinetobacter* Sp. By Transgenic Plant DNA Strictly Depends on Homologous Sequences in the Recipient Cells. *FEMS Microbiol. Lett.* 195 (2), 211–215. doi:10.1111/j.1574-6968.2001.tb10523.x

Deaville, E. R., and Maddison, B. C. (2005). Detection of Transgenic and Endogenous Plant DNA Fragments in the Blood, Tissues, and Digesta of Broilers. *J. Agric. Food Chem.* 53 (26), 10268–10275. doi:10.1021/jf051652f

Demanèche, S., Monier, J.-M., Dugat-Bony, E., and Simonet, P. (2011). Exploration of Horizontal Gene Transfer between Transplastomic Tobacco and Plant-Associated Bacteria. *FEMS Microbiol. Ecol.* 78 (1), 129–136. doi:10.1111/j.1574-6941.2011.01126.x

Demanèche, S., Sanguin, H., Poté, J., Navarro, E., Bernillon, D., Mavingui, P., et al. (2008). Antibiotic-resistant Soil Bacteria in Transgenic Plant Fields. *Proc. Natl. Acad. Sci. U. S. A.* 105 (10), 3957–3962. doi:10.1073/pnas.0800072105

Deni, J., Message, B., Chioccioli, M., and Tepfer, D. (2005). Unsuccessful Search for DNA Transfer from Transgenic Plants to Bacteria in the Intestine of the Tobacco Horn Worm, *Manduca Sexta*. *Transgenic Res.* 14 (2), 207–215. doi:10.1007/s11248-004-6701-z

Domingues, S., Nielsen, K. M., and da Silva, G. J. (2012). Various Pathways Leading to the Acquisition of Antibiotic Resistance by Natural Transformation. *Mob. Genet. Elem.* 2 (6), 257–260. doi:10.4161/mge.23089

Drezen, J.-M., Josse, T., Bézier, A., Gauthier, J., Huguet, E., and Herniou, E. A. (2017). Impact of Lateral Transfers on the Genomes of Lepidoptera. *Genes.* 8 (11), 315. doi:10.3390/genes8110315

Dröge, M., Pühler, A., and Selbitschka, W. (1998). Horizontal Gene Transfer as a Biosafety Issue: A Natural Phenomenon of Public Concern. *J. Biotechnol.* 64 (1), 75–90. doi:10.1016/S0168-1656(98)00105-9

Dunning Hotopp, J. C., Clark, M. E., Oliveira, D. C., Foster, J. M., Fischer, P., Munoz Torres, M. C., et al. (2007). Widespread Lateral Gene Transfer from Intracellular Bacteria to Multicellular Eukaryotes. *Science* 317 (5845), 1753–1756. doi:10.1126/science.1142490

Dunning, L. T., Olofsson, J. K., Parisod, C., Choudhury, R. R., Moreno-Villena, J. J., Yang, Y., et al. (2019). Lateral Transfers of Large DNA Fragments Spread Functional Genes Among Grasses. *Proc. Natl. Acad. Sci. U. S. A.* 116 (10), 4416–4425. doi:10.1073/pnas.1810031116

Dupont, P.-Y., and Cox, M. P. (2017). Genomic Data Quality Impacts Automated Detection of Lateral Gene Transfer in Fungi. *G3 Genes.|Genomes|Genetics* 7 (4), 1301–1314. doi:10.1534/g3.116.038448

EFSA, Gennaro, A., Gomes, A., Herman, L., Nogue, F., and Papadopoulou, N. (2017). Explanatory note on DNA sequence similarity searches in the context of the assessment of horizontal gene transfer from plants to microorganisms. *EFSA Support. Publ.* 14 (7), 1273E. doi:10.2903/sp.efsa.2017.EN-1273

Fournier, G. P., Andam, C. P., and Gogarten, J. P. (2015). Ancient Horizontal Gene Transfer and the Last Common Ancestors. *BMC Evol. Biol.* 15 (1), 70. doi:10.1186/s12862-015-0350-0

Fuentes, I., Stegemann, S., Golczyk, H., Karcher, D., and Bock, R. (2014). Horizontal Genome Transfer as an Asexual Path to the Formation of New Species. *Nature* 511 (7508), 232–235. doi:10.1038/nature13291

Gao, C., Ren, X., Mason, A. S., Liu, H., Xiao, M., Li, J., et al. (2014). Horizontal Gene Transfer in Plants. *Funct. Integr. Genomics* 14 (1), 23–29. doi:10.1007/s10142-013-0345-0

Garcia, P. R. (2006). Directive 2001/18/EC on the Deliberate Release into the Environment of GMOs: An Overview and the Main Provisions for Placing on the Market. *J. Eur. Environ. Plan. Law* 3 (1), 3–12. doi:10.1163/187601006X00029

Gardner, C. M., Volkoff, S. J., and Gunsch, C. K. (2019). Examining the Behavior of Crop-Derived Antibiotic Resistance Genes in Anaerobic Sludge Batch Reactors under Thermophilic Conditions. *Biotechnol. Bioeng.* 116 (11), 3063–3071. doi:10.1002/bit.27134

Garnatje, T., Garcia, S., Vilatersana, R., and Vallès, J. (2006). Genome Size Variation in the Genus *Carthamus* (Asteraceae, Cardueae): Systematic Implications and Additive Changes during Allopolyploidization. *Ann. Bot.* 97 (3), 461–467. doi:10.1093/aob/mcj050

Gebhard, F., and Smalla, K. (1999). Monitoring Field Releases of Genetically Modified Sugar Beets for Persistence of Transgenic Plant DNA and Horizontal Gene Transfer. *FEMS Microbiol. Ecol.* 28 (3), 261–272. doi:10.1111/j.1574-6941.1999.tb00581.x

Gelvin, S. B. (2003). *Agrobacterium*-mediated Plant Transformation: the Biology behind the "Gene-jockeying" Tool. *Microbiol. Mol. Biol. Rev.* 67 (1), 16–37. doi:10.1128/mmbr.67.1.16-37.2003

Gelvin, S. B. (2017). Integration of *Agrobacterium* T-DNA into the Plant Genome. *Annu. Rev. Genet.* 51 (1), 195–217. doi:10.1146/annurev-genet-120215-035320

EFSAGennaro, A., Gomes, A., Herman, L., Nogue, F., Papadopoulou, N., et al. (2017). Explanatory Note on DNA Sequence Similarity Searches in the Context of the Assessment of Horizontal Gene Transfer from Plants to Microorganisms. *EFSA Support. Publ.* 14 (7), 1273E. doi:10.2903/sp.efsa.2017.EN-1273

Ghoshal, K., Theilmann, J., Reade, R., Maghodia, A., and Rochon, D. A. (2015). Encapsidation of Host RNAs by *Cucumber Necrosis Virus* Coat Protein during Both Agroinfiltration and Infection. *J. Virol.* 89 (21), 10748–10761. doi:10.1128/jvi.01466-15

Gladyshev, E. A., Meselson, M., and Arkhipova, I. R. (2008). Massive Horizontal Gene Transfer in Bdelloid Rotifers. *Science* 320 (5880), 1210–1213. doi:10.1126/science.1156407

Graham, L. A., Lougheed, S. C., Ewart, K. V., and Davies, P. L. (2008). Lateral Transfer of a Lectin-like Antifreeze Protein Gene in Fishes. *PLOS ONE* 3 (7), e2616. doi:10.1371/journal.pone.0002616

Grbic, M., Van Leeuwen, T., Clark, R. M., Rombauts, S., Rouze, P., Grbic, V., et al. (2011). The Genome of *Tetranychus Urticae* Reveals Herbivorous Pest Adaptations. *Nature* 479 (7374), 487–492. doi:10.1038/nature10640

Gurdon, C., Svab, Z., Feng, Y., Kumar, D., and Maliga, P. (2016). Cell-to-cell Movement of Mitochondria in Plants. *Proc. Natl. Acad. Sci. U. S. A.* 113 (12), 3395–3400. doi:10.1073/pnas.1518644113

Haegeman, A., Jones, J. T., and Danchin, E. G. J. (2011). Horizontal Gene Transfer in Nematodes: A Catalyst for Plant Parasitism? *Mol. Plant-Microbe Interact.* 24 (8), 879–887. doi:10.1094/mpmi-03-11-0055

Hawes, M. C., Curlango-Rivera, G., Xiong, Z., and Kessler, J. O. (2012). Roles of Root Border Cells in Plant Defense and Regulation of Rhizosphere Microbial Populations by Extracellular DNA 'trapping. *Plant Soil* 355 (1), 1–16. doi:10.1007/s11104-012-1218-3

Hecht, M. M., Nitz, N., Araujo, P. F., Sousa, A. O., Rosa, A. d. C., Gomes, D. A., et al. (2010). Inheritance of DNA Transferred from American Trypanosomes to Human Hosts. *PLOS ONE* 5 (2), e9181. doi:10.1371/journal.pone.0009181

Heck, G. R., CaJacob, C. A., and Padgette, S. R. (2003). "Discovery, Development, and Commercialization of Roundup Ready® Crops," in *Plant Biotechnology 2002 and beyond: Proceedings of the 10th IAPTC&B Congress June 23–28, 2002 Orlando, Florida, U.S.A.I.K. Vasil.* (Dordrecht: Springer Netherlands), 139–142.

Hendriksma, H. P., Kuting, M., Hartel, S., Nather, A., Dohrmann, A. B., Steffan-Dewenter, I., et al. (2013). Effect of Stacked Insecticidal Cry Proteins from Maize Pollen on Nurse Bees (*Apis mellifera Carnica*) and Their Gut Bacteria. *PLOS ONE* 8 (3), e59589. doi:10.1371/journal.pone.0059589

Hibdige, S. G. S., Raimondeau, P., Christin, P.-A., and Dunning, L. T. (2021). Widespread Lateral Gene Transfer Among Grasses. *New Phytol.* 230 (6), 2474–2486. doi:10.1111/nph.17328

Hiltunen, T., Virta, M., and Laine, A.-L. (2017). Antibiotic Resistance in the Wild: an Eco-Evolutionary Perspective. *Phil. Trans. R. Soc. B* 372 (1712), 20160039. doi:10.1098/rstb.2016.0039

Hily, J.-M., Demanèche, S., Poulicard, N., Tannières, M., Djennane, S., Beuve, M., et al. (2018). Metagenomic-based Impact Study of Transgenic Grapevine Rootstock on its Associated Virome and Soil Bacteriome. *Plant Biotechnol. J.* 16 (1), 208–220. doi:10.1111/pbi.12761

Hohlweg, U., and Doerfler, W. (2001). On the Fate of Plant or Other Foreign Genes upon the Uptake in Food or after Intramuscular Injection in Mice. *Mol. Gen. Genomics* 265 (2), 225–233. doi:10.1007/s004380100450

Huang, J. (2013). Horizontal Gene Transfer in Eukaryotes: the Weak-Link Model. *BioEssays* 35 (10), 868–875. doi:10.1002/bies.201300007

Huddleston, J. R. (2014). Horizontal Gene Transfer in the Human Gastrointestinal Tract: Potential Spread of Antibiotic Resistance Genes. *Infect. Drug Resist.* 7, 167–176. doi:10.2147/IDR.S48820

Hülter, N., and Wackernagel, W. (2008). Double Illegitimate Recombination Events Integrate DNA Segments through Two Different Mechanisms during Natural Transformation of *Acinetobacter Baylyi*. *Mol. Microbiol.* 67 (5), 984–995. doi:10.1111/j.1365-2958.2007.06096.x

Hultman, J., Tamminen, M., Pärnänen, K., Cairns, J., Karkman, A., and Virta, M. (2018). Host Range of Antibiotic Resistance Genes in Wastewater Treatment Plant Influent and Effluent. *FEMS Microbiol. Ecol.* 94 (4). doi:10.1093/femsec/fiy038

Jennings, J. C., Kolwyck, D. C., Kays, S. B., Whetsell, A. J., Surber, J. B., Cromwell, G. L., et al. (2003). Determining whether Transgenic and Endogenous Plant DNA and Transgenic Protein Are Detectable in Muscle from Swine Fed Roundup Ready Soybean Meal1, 2, 3. *J. Animal Sci.* 81 (6), 1447–1455. doi:10.2527/2003.8161447x

Jonas, D. A., Elmadfa, I., Engel, K. H., Heller, K. J., Kozianowski, G., König, A., et al. (2001). Safety Considerations of DNA in Food. *Ann. Nutr. Metab.* 45 (6), 235–254. doi:10.1159/000046734

Kay, E., Vogel, T. M., Bertolla, F., Nalin, R., and Simonet, P. (2002). *In Situ* transfer of Antibiotic Resistance Genes from Transgenic (Transplastomic) Tobacco Plants to Bacteria. *Appl. Environ. Microbiol.* 68 (7), 3345–3351. doi:10.1128/aem.68.7.3345-3351.2002

Keeling, P. J. (2009). Functional and Ecological Impacts of Horizontal Gene Transfer in Eukaryotes. *Curr. Opin. Genet. Dev.* 19 (6), 613–619. doi:10.1016/j.gde.2009.10.001

Keeling, P. J., and Palmer, J. D. (2008). Horizontal Gene Transfer in Eukaryotic Evolution. *Nat. Rev. Genet.* 9 (8), 605–618. doi:10.1038/nrg2386

Keese, P. (2008). Risks from GMOs Due to Horizontal Gene Transfer. *Environ. Biosaf. Res.* 7 (3), 123–149. doi:10.1051/ebr:2008014

Korwin-Kossakowska, A., Sartowska, K., Tomczyk, G., Prusak, B., and Sender, G. (2016). Health Status and Potential Uptake of Transgenic DNA by Japanese Quail Fed Diets Containing Genetically Modified Plant Ingredients over 10 Generations. *Br. Poult. Sci.* 57 (3), 415–423. doi:10.1080/00071668.2016.1162281

Kyndt, T., Quispe, D., Zhai, H., Jarret, R., Ghislain, M., Liu, Q., et al. (2015). The Genome of Cultivated Sweet Potato Contains *Agrobacterium* T-DNAs with Expressed Genes: An Example of a Naturally Transgenic Food Crop. *Proc. Natl. Acad. Sci. U. S. A.* 112 (18), 5844–5849. doi:10.1073/pnas.1419685112

Lang, A. S., Zhaxybayeva, O., and Beatty, J. T. (2012). Gene Transfer Agents: Phage-like Elements of Genetic Exchange. *Nat. Rev. Microbiol.* 10 (7), 472–482. doi:10.1038/nrmicro2802

Latham, J. R., Love, M., and Hilbeck, A. (2017). The Distinct Properties of Natural and GM Cry Insecticidal Proteins. *Biotechnol. Genet. Eng. Rev.* 33 (1), 62–96. doi:10.1080/02648725.2017.1357295

Lerner, A., Matthias, T., and Aminov, R. (2017). Potential Effects of Horizontal Gene Exchange in the Human Gut. *Front. Immunol.* 8 (1630). doi:10.3389/fimmu.2017.01630

Li, F.-W., Villarreal, J. C., Kelly, S., Rothfels, C. J., Melkonian, M., Frangedakis, E., et al. (2014). Horizontal Transfer of an Adaptive Chimeric Photoreceptor from Bryophytes to Ferns. *Proc. Natl. Acad. Sci. U. S. A.* 111 (18), 6672–6677. doi:10.1073/pnas.1319929111

Li, M., Zhao, J., Tang, N., Sun, H., and Huang, J. (2018). Horizontal Gene Transfer from Bacteria and Plants to the Arbuscular Mycorrhizal Fungus *Rhizophagus Irregularis*. *Front. Plant Sci.* 9 (701). doi:10.3389/fpls.2018.00701

Lin, T., Chen, Z., Usha, R., Stauffacher, C. V., Dai, J.-B., Schmidt, T., et al. (1999). The Refined Crystal Structure of Cowpea Mosaic Virus at 2.8 Å Resolution. *Virology* 265 (1), 20–34. doi:10.1006/viro.1999.0038

Losey, J. E., Harmon, J., Ballantyne, F., and Brown, C. (1997). A Polymorphism Maintained by Opposite Patterns of Parasitism and Predation. *Nature* 388 (6639), 269–272. doi:10.1038/40849

Ma, B. L., Blackshaw, R. E., Roy, J., and He, T. (2011). Investigation on Gene Transfer from Genetically Modified Corn (*Zea mays* L.) Plants to Soil Bacteria. *J. Environ. Sci. Health, Part B* 46 (7), 590–599. doi:10.1080/03601234.2011.586598

Mahelka, V., Krak, K., Kopecký, D., Fehrer, J., Šafář, J., Bartoš, J., et al. (2017). Multiple Horizontal Transfers of Nuclear Ribosomal Genes between Phylogenetically Distinct Grass Lineages. *Proc. Natl. Acad. Sci. U. S. A.* 114 (7), 1726–1731. doi:10.1073/pnas.1613375114

Maliga, P., and Bock, R. (2011). Plastid Biotechnology: Food, Fuel, and Medicine for the 21st Century. *Plant Physiol.* 155 (4), 1501–1510. doi:10.1104/pp.110.170969

Mao, J., and Lu, T. (2016). Population-dynamic Modeling of Bacterial Horizontal Gene Transfer by Natural Transformation. *Biophysical J.* 110 (1), 258–268. doi:10.1016/j.bpj.2015.11.033

Matveeva, T. V., and Otten, L. (2019). Widespread Occurrence of Natural Genetic Transformation of Plants by *Agrobacterium*. *Plant Mol. Biol.* 101, 415–437. doi:10.1007/s11103-019-00913-y

Mazza, R., Soave, M., Morlacchini, M., Piva, G., and Marocco, A. (2005). Assessing the Transfer of Genetically Modified DNA from Feed to Animal Tissues. *Transgenic Res.* 14 (5), 775–784. doi:10.1007/s11248-005-0009-5

McAdams, H. H., Srinivasan, B., and Arkin, A. P. (2004). The Evolution of Genetic Regulatory Systems in Bacteria. *Nat. Rev. Genet.* 5 (3), 169–178. doi:10.1038/nrg1292

McDonald, M. C., Taranto, A. P., Hill, E., Schwessinger, B., Liu, Z., Simpfendorfer, S., et al. (2019). Transposon-mediated Horizontal Transfer of the Host-specific Virulence Protein ToxA between Three Fungal Wheat Pathogens. *mBio* 10 (5), e01515–01519. doi:10.1128/mBio.01515-19

Mitreva, M., Smant, G., and Helder, J. (2009). "Role of Horizontal Gene Transfer in the Evolution of Plant Parasitism Among Nematodes," in *Horizontal Gene Transfer: Genomes in Flux*. Editors M. B. Gogarten, J. P. Gogarten, and L. C. Olendzenski (Totowa, NJ: Humana Press), 517–535.

Miyashita, H., Kuroki, Y., Kretsinger, R. H., and Matsushima, N. (2013). Horizontal Gene Transfer of Plant-specific Leucine-Rich Repeats between Plants and Bacteria. *Nat. Sci.* 5 (5), 580–598. doi:10.4236/ns.2013.55074

Mohr, K. I., and Tebbe, C. C. (2007). Field Study Results on the Probability and Risk of a Horizontal Gene Transfer from Transgenic Herbicide-Resistant Oilseed Rape Pollen to Gut Bacteria of Bees. *Appl. Microbiol. Biotechnol.* 75 (3), 573–582. doi:10.1007/s00253-007-0846-7

Monier, J.-M., Bernillon, D., Kay, E., Faugier, A., Rybalka, O., Dessaux, Y., et al. (2007). Detection of Potential Transgenic Plant DNA Recipients Among Soil Bacteria. *Environ. Biosaf. Res.* 6 (1-2), 71–83. doi:10.1051/ebr:2007036

Moran, N. A., and Jarvik, T. (2010). Lateral Transfer of Genes from Fungi Underlies Carotenoid Production in Aphids. *Science* 328 (5978), 624–627. doi:10.1126/science.1187113

Nadal, A., De Giacomo, M., Einspanier, R., Kleter, G., Kok, E., McFarland, S., et al. (2018). Exposure of Livestock to GM Feeds: Detectability and Measurement. *Food Chem. Toxicol.* 117, 13–35. doi:10.1016/j.fct.2017.08.032

Nawaz, M. A., Mesnage, R., Tsatsakis, A. M., Golokhvast, K. S., Yang, S. H., Antoniou, M. N., et al. (2019). Addressing Concerns over the Fate of DNA Derived from Genetically Modified Food in the Human Body: A Review. *Food Chem. Toxicol.* 124, 423–430. doi:10.1016/j.fct.2018.12.030

Nemeth, A., Wurz, A., Artim, L., Charlton, S., Dana, G., Glenn, K., et al. (2004). Sensitive PCR Analysis of Animal Tissue Samples for Fragments of Endogenous and Transgenic Plant DNA. *J. Agric. Food Chem.* 52 (20), 6129–6135. doi:10.1021/jf049567f

Netherwood, T., Martín-Orúe, S. M., O'Donnell, A. G., Gockling, S., Graham, J., Mathers, J. C., et al. (2004). Assessing the Survival of Transgenic Plant DNA in the Human Gastrointestinal Tract. *Nat. Biotechnol.* 22, 204–209. doi:10.1038/nbt934

Nicolia, A., Manzo, A., Veronesi, F., and Rosellini, D. (2014). An Overview of the Last 10 Years of Genetically Engineered Crop Safety Research. *Crit. Rev. Biotechnol.* 34 (1), 77–88. doi:10.3109/07388551.2013.823595

Nielsen, K. M., Bones, A. M., Smalla, K., and van Elsas, J. D. (1998). Horizontal Gene Transfer from Transgenic Plants to Terrestrial Bacteria – a Rare Event? *FEMS Microbiol. Rev.* 22 (2), 79–103. doi:10.1016/s0168-6445(98)00009-6

Nikoh, N., and Nakabachi, A. (2009). Aphids Acquired Symbiotic Genes *via* Lateral Gene Transfer. *BMC Biol.* 7, 12. doi:10.1186/1741-7007-7-12

Nikoh, N., Tanaka, K., Shibata, F., Kondo, N., Hizume, M., Shimada, M., et al. (2008). *Wolbachia* Genome Integrated in an Insect Chromosome: Evolution and Fate of Laterally Transferred Endosymbiont Genes. *Genome Res.* 18 (2), 272–280. doi:10.1101/gr.7144908

Nikolaidis, N., Doran, N., and Cosgrove, D. J. (2013). Plant Expansins in Bacteria and Fungi: Evolution by Horizontal Gene Transfer and Independent Domain Fusion. *Mol. Biol. Evol.* 31 (2), 376–386. doi:10.1093/molbev/mst206

Niu, L., Ma, W., Lei, C., Jurat-Fuentes, J. L., and Chen, L. (2017). Herbicide and Insect Resistant Bt Cotton Pollen Assessment Finds No Detrimental Effects on Adult Honey Bees. *Environ. Pollut.* 230, 479–485. doi:10.1016/j.envpol.2017.06.094

Noon, J. B., and Baum, T. J. (2016). Horizontal Gene Transfer of Acetyltransferases, Invertases and Chorismate Mutases from Different Bacteria to Diverse Recipients. *BMC Evol. Biol.* 16, 74. doi:10.1186/s12862-016-0651-y

Nordgård, L., Brusetti, L., Raddadi, N., Traavik, T., Averhoff, B., and Nielsen, K. M. (2012). An Investigation of Horizontal Transfer of Feed Introduced DNA to the Aerobic Microbiota of the Gastrointestinal Tract of Rats. *BMC Res. Notes* 5 (1), 170. doi:10.1186/1756-0500-5-170

OGTR (2020). *Gene Technology Regulations 2001*. Canberra: Office of Parliamentary Counsel.

OGTR (2013). *Risk Analysis Framework 2013*. Canberra: Office of the Gene Technology Regulator.

O'Sullivan, G. M., Philips, J. G., Mitchell, H. J., Dornbusch, M., and Rasko, J. E. J. (2022). 20 Years of Legislation - How Australia Has Responded to the Challenge of Regulating Genetically Modified Organisms in the Clinic. *Front. Med.* 9, 883434. doi:10.3389/fmed.2022.883434

Parrott, W., Chassy, B., Ligon, J., Meyer, L., Petrick, J., Zhou, J., et al. (2010). Application of Food and Feed Safety Assessment Principles to Evaluate Transgenic Approaches to Gene Modulation in Crops. *Food Chem. Toxicol.* 48 (7), 1773–1790. doi:10.1016/j.fct.2010.04.017

Pettersen, A. K., Bohn, T., Primicerio, R., Shorten, P. R., Soboleva, T. K., and Nielsen, K. M. (2005). Modeling Suggests Frequency Estimates Are Not Informative for Predicting the Long-Term Effect of Horizontal Gene Transfer in Bacteria. *Environ. Biosaf. Res.* 4 (4), 223–233. doi:10.1051/ebr:2006008

Philips, J. G., Naim, F., Lorenc, M. T., Dudley, K. J., Hellens, R. P., and Waterhouse, P. M. (2017). The Widely Used *Nicotiana Benthamiana* 16c Line Has an Unusual T-DNA Integration Pattern Including a Transposon Sequence. *PLOS ONE* 12 (2), e0171311. doi:10.1371/journal.pone.0171311

Pilate, G., Allona, I., Boerjan, W., Déjardin, A., Fladung, M., Gallardo, F., et al. (2016). "Lessons from 25 Years of GM Tree Field Trials in Europe and Prospects for the Future," in *Biosafety of Forest Transgenic Trees: Improving the Scientific Basis for Safe Tree Development and Implementation of EU Policy Directives*. Editors C. Vettori, F. Gallardo, H. Häggman, V. Kazana, F. Migliacci, G. Pilate, et al. (Dordrecht: Springer Netherlands), 67–100.

Pontiroli, A., Ceccherini, M.-T., Poté, J., Wildi, W., Kay, E., Nannipieri, P., et al. (2010). Long-term Persistence and Bacterial Transformation Potential of Transplastomic Plant DNA in Soil. *Res. Microbiol.* 161 (5), 326–334. doi:10.1016/j.resmic.2010.04.009

Pontiroli, A., Rizzi, A., Simonet, P., Daffonchio, D., Vogel, T. M., and Monier, J.-M. (2009). Visual Evidence of Horizontal Gene Transfer between Plants and Bacteria in the Phytosphere of Transplastomic Tobacco. *Appl. Environ. Microbiol.* 75 (10), 3314–3322. doi:10.1128/AEM.02632-08

Pontiroli, A., Simonet, P., Frostegard, A., Vogel, T. M., and Monier, J.-M. (2007). Fate of Transgenic Plant DNA in the Environment. *Environ. Biosaf. Res.* 6 (1-2), 15–35. doi:10.1051/ebr:2007037

Poté, J., Ackermann, R., and Wildi, W. (2009). Plant Leaf Mass Loss and DNA Release in Freshwater Sediments. *Ecotoxicol. Environ. Saf.* 72 (5), 1378–1383. doi:10.1016/j.ecoenv.2009.04.010

Prentice, H. C., Li, Y., Lönn, M., Tunlid, A., and Ghatnekar, L. (2015). A Horizontally Transferred Nuclear Gene Is Associated with Microhabitat Variation in a Natural Plant Population. *Proc. R. Soc. B* 282 (1821), 20152453. doi:10.1098/rspb.2015.2453

Qiu, H., Cai, G., Luo, J., Bhattacharya, D., and Zhang, N. (2016). Extensive Horizontal Gene Transfers between Plant Pathogenic Fungi. *BMC Biol.* 14 (1), 41. doi:10.1186/s12915-016-0264-3

Quispe-Huamanquispe, D. G., Gheysen, G., and Kreuze, J. F. (2017). Horizontal Gene Transfer Contributes to Plant Evolution: The Case of *Agrobacterium* T-DNAs. *Front. Plant Sci.* 8, 2015. doi:10.3389/fpls.2017.02015

Ravenhall, M., Škunca, N., Lassalle, F., and Dessimoz, C. (2015). Inferring Horizontal Gene Transfer. *PLoS Comput. Biol.* 11 (5), e1004095. doi:10.1371/journal.pcbi.1004095

Raz, Y., and Tannenbaum, E. (2010). The Influence of Horizontal Gene Transfer on the Mean Fitness of Unicellular Populations in Static Environments. *Genetics* 185 (1), 327–337. doi:10.1534/genetics.109.113613

Rice, D. W., Alverson, A. J., Richardson, A. O., Young, G. J., Sanchez-Puerta, M. V., Munzinger, J., et al. (2013). Horizontal Transfer of Entire Genomes *via* Mitochondrial Fusion in the Angiosperm *Amborella*. *Science* 342 (6165), 1468–1473. doi:10.1126/science.1246275

Richards, T. A., Soanes, D. M., Foster, P. G., Leonard, G., Thornton, C. R., and Talbot, N. J. (2009). Phylogenomic Analysis Demonstrates a Pattern of Rare and Ancient Horizontal Gene Transfer between Plants and Fungi. *Plant Cell* 21 (7), 1897–1911. doi:10.1105/tpc.109.065805

Richards, T. A., Soanes, D. M., Jones, M. D., Vasieva, O., Leonard, G., Paszkiewicz, K., et al. (2011). Horizontal Gene Transfer Facilitated the Evolution of Plant Parasitic Mechanisms in the Oomycetes. *Proc. Natl. Acad. Sci. U. S. A.* 108 (37), 15258–15263. doi:10.1073/pnas.1105100108

Richardson, A. O., and Palmer, J. D. (2007). Horizontal Gene Transfer in Plants. *J. Exp. Bot.* 58 (1), 1–9. doi:10.1093/jxb/erl148

Rigden, J. E., Dry, I. B., Krake, L. R., and Rezaian, M. A. (1996). Plant Virus DNA Replication Processes in *Agrobacterium*: Insight into the Origins of Geminiviruses? *Proc. Natl. Acad. Sci. U. S. A.* 93 (19), 10280–10284. doi:10.1073/pnas.93.19.10280

Rizzi, A., Raddadi, N., Sorlini, C., Nordgrd, L., Nielsen, K. M., and Daffonchio, D. (2012). The Stability and Degradation of Dietary DNA in the Gastrointestinal Tract of Mammals: Implications for Horizontal Gene Transfer and the Biosafety of GMOs. *Crit. Rev. Food Sci. Nutr.* 52 (2), 142–161. doi:10.1080/10408398.2010.499480

Rose, A. S., Bradley, A. R., Valasatava, Y., Duarte, J. M., Prlić, A., and Rose, P. W. (2018). NGL Viewer: Web-Based Molecular Graphics for Large Complexes. *Bioinformatics* 34 (21), 3755–3758. doi:10.1093/bioinformatics/bty419

Rossi, F., Morlacchini, M., Fusconi, G., Pietri, A., Mazza, R., and Piva, G. (2005). Effect of Bt Corn on Broiler Growth Performance and Fate of Feed-Derived DNA in the Digestive Tract. *Poult. Sci.* 84 (7), 1022–1030. doi:10.1093/ps/84.7.1022

Rumpho, M. E., Worful, J. M., Lee, J., Kannan, K., Tyler, M. S., Bhattacharya, D., et al. (2008). Horizontal Gene Transfer of the Algal Nuclear Gene *psbO* to the Photosynthetic Sea Slug *Elysia Chlorotica*. *Proc. Natl. Acad. Sci. U. S. A.* 105 (46), 17867–17871. doi:10.1073/pnas.0804968105

Sakamoto, W., and Takami, T. (2018). Chloroplast DNA Dynamics: Copy Number, Quality Control and Degradation. *Plant Cell Physiology* 59 (6), 1120–1127. doi:10.1093/pcp/pcy084

Sanchez-Puerta, M. V., Edera, A., Gandini, C. L., Williams, A. V., Howell, K. A., Nevill, P. G., et al. (2019). Genome-scale Transfer of Mitochondrial DNA from Legume Hosts to the Holoparasite *Lophophytum Mirabile* (Balanophoraceae). *Mol. Phylogenetics Evol.* 132, 243–250. doi:10.1016/j.ympev.2018.12.006

Sand, K. K., and Jelavić, S. (2018). Mineral Facilitated Horizontal Gene Transfer: A New Principle for Evolution of Life? *Front. Microbiol.* 9 (2217). doi:10.3389/fmicb.2018.02217

Schneider, C. L. (2017). "Bacteriophage-Mediated Horizontal Gene Transfer: Transduction," in *Bacteriophages: Biology, Technology, Therapy*. Editors D. Harper, S. Abedon, B. Burrowes, and M. McConville (Cham: Springer International Publishing), 1–42.

Schönknecht, G., Weber, A. P. M., and Lercher, M. J. (2014). Horizontal Gene Acquisitions by Eukaryotes as Drivers of Adaptive Evolution. *BioEssays* 36 (1), 9–20. doi:10.1002/bies.201300095

Schreiber, M., Stein, N., and Mascher, M. (2018). Genomic Approaches for Studying Crop Evolution. *Genome Biol.* 19 (1), 140. doi:10.1186/s13059-018-1528-8

Schubbert, R., Hohlweg, U., Renz, D., and Doerfler, W. (1998). On the Fate of Orally Ingested Foreign DNA in Mice: Chromosomal Association and Placental Transmission to the Fetus. *Mol. Gen. Genet.* 259 (6), 569–576. doi:10.1007/s004380050850

Schubbert, R., Renz, D., Schmitz, B., and Doerfler, W. (1997). Foreign (M13) DNA Ingested by Mice Reaches Peripheral Leukocytes, Spleen, and Liver *via* the Intestinal Wall Mucosa and Can Be Covalently Linked to Mouse DNA. *Proc. Natl. Acad. Sci. U. S. A.* 94 (3), 961–966. doi:10.1073/pnas.94.3.961

Sharma, R., Damgaard, D., Alexander, T. W., Dugan, M. E., Aalhus, J. L., Stanford, K., et al. (2006). Detection of Transgenic and Endogenous Plant DNA in Digesta and Tissues of Sheep and Pigs Fed Roundup Ready Canola Meal. *J. Agric. Food Chem.* 54 (5), 1699–1709. doi:10.1021/jf052459o

Shen, J., Zhang, Y., Havey, M. J., and Shou, W. (2019). Copy Numbers of Mitochondrial Genes Change during Melon Leaf Development and Are Lower

Than the Numbers of Mitochondria. *Hortic. Res.* 6 (1), 95. doi:10.1038/s41438-019-0177-8

Shinozuka, H., Hettiarachchige, I. K., Shinozuka, M., Cogan, N. O. I., Spangenberg, G. C., Cocks, B. G., et al. (2017). Horizontal Transfer of a SS-1, 6-glucanase Gene from an Ancestral Species of Fungal Endophyte to a Cool-Season Grass Host. *Sci. Rep.* 7 (1), 9024. doi:10.1038/s41598-017-07886-2

Sieber, K. B., Bromley, R. E., and Dunning Hotopp, J. C. (2017). Lateral Gene Transfer between Prokaryotes and Eukaryotes. *Exp. Cell Res.* 358 (2), 421–426. doi:10.1016/j.yexcr.2017.02.009

Sieradzki, Z., Mazur, M., Kwiatek, K., Świątkiewicz, S., Świątkiewicz, M., Koreleski, J., et al. (2013). Assessing the Possibility of Genetically Modified DNA Transfer from GM Feed to Broiler, Laying Hen, Pig and Calf Tissues. *Pol. J. Veterinary Sci.* 16 (3), 435–441. doi:10.2478/pjvs-2013-0061

Sinn, B. T., and Barrett, C. F. (2019). Ancient Mitochondrial Gene Transfer between Fungi and the Orchids. *Mol. Biol. Evol.* 37, 44–57. doi:10.1093/molbev/msz198

Soanes, D., and Richards, T. A. (2014). Horizontal Gene Transfer in Eukaryotic Plant Pathogens. *Annu. Rev. Phytopathol.* 52, 583–614. doi:10.1146/annurev-phyto-102313-050127

Soucy, S. M., Huang, J., and Gogarten, J. P. (2015). Horizontal Gene Transfer: Building the Web of Life. *Nat. Rev. Genet.* 16 (8), 472–482. doi:10.1038/nrg3962

Stegemann, S., and Bock, R. (2009). Exchange of Genetic Material between Cells in Plant Tissue Grafts. *Science* 324 (5927), 649–651. doi:10.1126/science.1170397

Stegemann, S., Keuthe, M., Greiner, S., and Bock, R. (2012). Horizontal Transfer of Chloroplast Genomes between Plant Species. *Proc. Natl. Acad. Sci. U. S. A.* 109 (7), 2434–2438. doi:10.1073/pnas.1114076109

Świątkiewicz, M., Bednarek, D., Markowski, J., Hanczakowska, E., and Kwiate, K. (2013). "Effect of Feeding Genetically Modified Maize and Soybean Meal to Sows on Their Reproductive Traits, Haematological Indices and Offspring Performance," in *Bulletin of the Veterinary Institute in Pulawy*.

Thyssen, G., Svab, Z., and Maliga, P. (2012). Cell-to-cell Movement of Plastids in Plants. *Proc. Natl. Acad. Sci. U. S. A.* 109 (7), 2439–2443. doi:10.1073/pnas.1114297109

Tothova, T., Sobekova, A., Holovska, K., Legath, J., Pristas, P., and Javorsky, P. (2010). "Natural Glufosinate Resistance of Soil Microorganisms and GMO Safety," in *Open Life Sciences*.

Tsatsakis, A. M., Nawaz, M. A., Kouretas, D., Balias, G., Savolainen, K., Tutelyan, V. A., et al. (2017). Environmental Impacts of Genetically Modified Plants: A Review. *Environ. Res.* 156, 818–833. doi:10.1016/j.envres.2017.03.011

Ülker, B., Li, Y., Rosso, M. G., Logemann, E., Somssich, I. E., and Weisshaar, B. (2008). T-DNA–mediated Transfer of *Agrobacterium Tumefaciens* Chromosomal DNA into Plants. *Nat. Biotechnol.* 26 (9), 1015–1017. doi:10.1038/nbt.1491

Van Eenennaam, A. L., and Young, A. E. (2014). Prevalence and Impacts of Genetically Engineered Feedstuffs on Livestock Populations1. *J. Animal Sci.* 92 (10), 4255–4278. doi:10.2527/jas.2014-8124

van Elsas, J. D., Turner, S., and Bailey, M. J. (2003). Horizontal Gene Transfer in the Phytosphere. *New Phytol.* 157 (3), 525–537. doi:10.1046/j.1469-8137.2003.00697.x

Vogan, A. A., and Higgs, P. G. (2011). The Advantages and Disadvantages of Horizontal Gene Transfer and the Emergence of the First Species. *Biol. Direct* 6, 1. doi:10.1186/1745-6150-6-1

Walsh, M. C., Buzoianu, S. G., Gardiner, G. E., Rea, M. C., Gelencser, E., Janosi, A., et al. (2011). Fate of Transgenic DNA from Orally Administered Bt Mon810 Maize and Effects on Immune Response and Growth in Pigs. *PLOS ONE* 6 (11), e27177. doi:10.1371/journal.pone.0027177

Wickell, D. A., and Li, F.-W. (2019). On the Evolutionary Significance of Horizontal Gene Transfers in Plants. *New Phytol.* 225, 113–117. doi:10.1111/nph.16022

Wilcks, A., and Jacobsen, B. B. (2010). Lack of Detectable DNA Uptake by Transformation of Selected Recipients in Mono-Associated Rats. *BMC Res. Notes* 3 (1), 49. doi:10.1186/1756-0500-3-49

Woegerbauer, M., Zeinzinger, J., Gottsberger, R. A., Pascher, K., Hufnagl, P., Indra, A., et al. (2015). Antibiotic Resistance Marker Genes as Environmental Pollutants in GMO-Pristine Agricultural Soils in Austria. *Environ. Pollut.* 206, 342–351. doi:10.1016/j.envpol.2015.07.028

Wybouw, N., Pauchet, Y., Heckel, D. G., and Van Leeuwen, T. (2016). Horizontal Gene Transfer Contributes to the Evolution of Arthropod Herbivory. *Genome Biol. Evol.* 8 (6), 1785–1801. doi:10.1093/gbe/evw119

Wybouw, N., Van Leeuwen, T., and Dermauw, W. (2018). A Massive Incorporation of Microbial Genes into the Genome of *Tetranychus Urticae*, a Polyphagous Arthropod Herbivore. *Insect Mol. Biol.* 27 (3), 333–351. doi:10.1111/imb.12374

Xi, Z., Wang, Y., Bradley, R. K., Sugumaran, M., Marx, C. J., Rest, J. S., et al. (2013). Massive Mitochondrial Gene Transfer in a Parasitic Flowering Plant Clade. *PLoS Genet.* 9 (2), e1003265. doi:10.1371/journal.pgen.1003265

Xia, J., Guo, Z., Yang, Z., Han, H., Wang, S., Xu, H., et al. (2021). Whitefly Hijacks a Plant Detoxification Gene that Neutralizes Plant Toxins. *Cell* 184 (7), 1693–1705.e17. doi:10.1016/j.cell.2021.02.014

Yang, T., Chen, Y., Wang, X.-X., and Dai, C.-C. (2013). Plant Symbionts: Keys to the Phytosphere. *Symbiosis* 59 (1), 1–14. doi:10.1007/s13199-012-0190-2

Yang, Z., Wafula, E. K., Kim, G., Shahid, S., McNeal, J. R., Ralph, P. E., et al. (2019). Convergent Horizontal Gene Transfer and Cross-Talk of Mobile Nucleic Acids in Parasitic Plants. *Nat. Plants* 5 (9), 991–1001. doi:10.1038/s41477-019-0458-0

Yang, Z., Zhang, Y., Wafula, E. K., Honaas, L. A., Ralph, P. E., Jones, S., et al. (2016). Horizontal Gene Transfer Is More Frequent with Increased Heterotrophy and Contributes to Parasite Adaptation. *Proc. Natl. Acad. Sci. U. S. A.* 113 (45), E7010–E7019. doi:10.1073/pnas.1608765113

Yin, Z., Zhu, B., Feng, H., and Huang, L. (2016). Horizontal Gene Transfer Drives Adaptive Colonization of Apple Trees by the Fungal Pathogen *Valsa mali*. *Sci. Rep.* 6, 33129. doi:10.1038/srep33129

Yonemochi, C., Suga, K., Harada, C., and Hanazumi, M. (2010). Tevaluation of Transgenic Event CBH 351 (StarLink) Corn in Pig. *Animal Sci. J.* 81 (1), 94–101. doi:10.1111/j.1740-0929.2009.00718.x

Yoshida, S., Cui, S., Ichihashi, Y., and Shirasu, K. (2016). The Haustorium, a Specialized Invasive Organ in Parasitic Plants. *Annu. Rev. Plant Biol.* 67 (1), 643–667. doi:10.1146/annurev-arplant-043015-111702

Zhao, K., Ren, F., Han, F., Liu, Q., Wu, G., Xu, Y., et al. (2016). Edible Safety Assessment of Genetically Modified Rice T1C-1 for Sprague Dawley Rats through Horizontal Gene Transfer, Allergenicity and Intestinal Microbiota. *PLOS ONE* 11 (10), e0163352. doi:10.1371/journal.pone.0163352

Zhu, B. (2006). Degradation of Plasmid and Plant DNA in Water Microcosms Monitored by Natural Transformation and Real-Time Polymerase Chain Reaction (PCR). *Water Res.* 40 (17), 3231–3238. doi:10.1016/j.watres.2006.06.040

Zhu, B., Lou, M.-M., Xie, G.-L., Zhang, G.-Q., Zhou, X.-P., Li, B., et al. (2011). Horizontal Gene Transfer in Silkworm, *Bombyx mori*. *BMC Genomics* 12 (1), 248. doi:10.1186/1471-2164-12-248

Zhu, Y., Li, D., Wang, F., Yin, J., and Jin, H. (2004). Nutritional Assessment and Fate of DNA of Soybean Meal from Roundup Ready or Conventional Soybeans Using Rats. *Archives Animal Nutr.* 58 (4), 295–310. doi:10.1080/00039420412331273277

frontiers | Frontiers in Bioengineering and Biotechnology

TYPE Policy and Practice Reviews
PUBLISHED 27 September 2022
DOI 10.3389/fbioe.2022.946526

OPEN ACCESS

EDITED BY
Patricia Machado Bueno Fernandes,
Federal University of Espirito Santo,
Brazil

REVIEWED BY
Silas Pessini Rodrigues,
Federal University of Rio de Janeiro,
Brazil
José Aires Ventura,
Capixaba Institute for Research,
Technical Assistance and Rural
Extension (INCAPER), Brazil

*CORRESPONDENCE
Britte Bouchaut,
B.f.h.j.bouchaut@tudelft.nl

SPECIALTY SECTION
This article was submitted to Biosafety and Biosecurity,
a section of the journal
Frontiers in Bioengineering and Biotechnology

RECEIVED 17 May 2022
ACCEPTED 12 September 2022
PUBLISHED 27 September 2022

CITATION
Bouchaut B, de Vriend H and Asveld L (2022), Uncertainties and uncertain risks of emerging biotechnology applications: A social learning workshop for stakeholder communication.
Front. Bioeng. Biotechnol. 10:946526.
doi: 10.3389/fbioe.2022.946526

Uncertainties and uncertain risks of emerging biotechnology applications: A social learning workshop for stakeholder communication

Britte Bouchaut[1]*, Huib de Vriend[2] and Lotte Asveld[1]

[1]Delft University of Technology, Delft, Netherlands, [2]LIS Consult, Driebergen, Netherlands

Emerging applications of biotechnology such as new genomic techniques may give rise to new uncertainties and uncertain risks. Particularly the increased complexity and limited knowledge of possible risks associated with these new techniques, make it currently impossible to perform an adequate environmental risk assessment. As a result, there is a risk that such techniques don't get beyond experiments demonstrating the proof of principle, stifling their further development and implementation. To break free from this deadlock, we must be able to learn what such uncertainties and uncertain risks entail, and how they should be assessed to ensure safe further development. To shape a responsible learning environment to explore uncertainties and uncertain risks, we have organized five stakeholder workshops. By means of a case about the genetic engineering of plants' rhizosphere–an application abundant with uncertain risks–we identified tensions between different stakeholder groups and their different estimates of uncertainties and uncertain risks. Based upon derived insights, we developed a tool–a script for researchers to organize a stakeholder workshop–that enables a constructive discussion about emerging risks with a broad range of stakeholders. Thereby, the script provides a step-by-step approach to identify uncertainties, develop anticipatory strategies and adaptations in (experimental) research designs to lower or mitigate the earlier identified uncertainties, and helps to identify knowledge gaps for which (additional) risk research should be set up.

KEYWORDS

uncertain risks, safety, mutual learning, safe-by-design, responsibility, plant engineering

1 Introduction

Already in 2017, Hogervorst and colleagues pointed out various developments in biotechnology for which no adequate environmental risk assessment (ERA) could be performed at that moment (Hogervorst et al., 2017). In particular, the increasing complexity associated with new genomic techniques, and lack of knowledge thereof,

gives rise to debate on how to execute an ERA in such cases (Parisi and Rodriguez Cerezo, 2021). Currently, Europe's risk management regime regarding biotechnology seems to be one of compliance (Bouchaut and Asveld, 2021), which provides little room to learn what uncertainties and uncertain risks entail. With these types of uncertainty, we refer to the so-called "known unknowns"—instances of which we know we are missing information about the probability or severity of a harmful effect, or of which we do not know if there are any possible harmful effects to begin with (Aven and Renn, 2009). Due to the strong embeddedness and operationalization of the precautionary principle (PP), potentially having a risk involved is sufficient to take cost-effective measures to prevent environmental degradation. In other words; uncertainty does not justify inaction, or ultimately limits research (Brisman, 2011). However, these measures should be based on an examination of the potential benefits and costs, or lack of action, and be subject to review in the light of new scientific data (Commission of the European Communities, 2000).

The way the PP has been operationalized in Europe has resulted in a normative framework in which the biological safety protocol is currently subjected to a dilemma between safety and innovation. While it ensures safety on known and acceptable risks, it also hinders innovation as it stifles research with uncertainties involved. Indeed, present regulation based on the PP only allows very little room for learning about uncertainties and how to mitigate uncertain risks, and thus also whether uncertainties should be regarded as uncertain risks, and uncertain risks as (unacceptable) risks (van Asselt and Vos, 2006, van Asselt and Vos, 2008; Flage and Aven, 2015). In addition, learning being limited also results in maintaining a lack of knowledge regarding the potential benefits, which also creates a deadlock for reviewing earlier taken precautionary measures in the light of new knowledge.

To break free from this impasse, the process of ERA must create more room to learn what uncertainties and uncertain risks entail, and based on this information, define how to assess and regard these. But this learning may be complicated by differing perspectives from stakeholders on uncertainties and uncertain risks (Bouchaut and Asveld, 2020). So foremost, we need to increase mutual understanding of differing perspectives. A new approach to facilitating learning about uncertainties (both potential risks and benefits) would require extensive communication and mutual learning between various stakeholders. Although dependent on the partaking stakeholders' fields of expertise (e.g., technical, regulatory or societal domain), we must ensure that this learning is conducted in line with possible (societal) concerns and that any results are taken up swiftly by relevant stakeholders to allow for some form of adaptive risk management. The question that emerges from this is how to organize such a learning process with a variety of stakeholders. This paper's aim is therefore twofold: develop a tool that enables a learning process regarding emerging uncertain risks and uncertainties, and evaluate whether learning has occurred. To do so, we organized five stakeholder workshops with participants from a range of expertise (e.g., technical researchers, social scientists, risk assessors, policymakers), building upon the International Risk Governance Council (IRGC) framework and the notion of "social learning" by van de Poel (2017).

The importance of learning processes is acknowledged by the IRGC framework that provides guidelines for dealing with situations characterized by a mix of complexity, uncertainty and/or normative ambiguity (Renn and Walker, 2008; IRGC, 2017). Particularly the framework's first step, the pre-assessment, involves relevant stakeholder groups to capture differing perspectives on potential risks, their associated opportunities and potential strategies to address these (IRGC, 2017). For our workshops, we complemented the IRGC's pre-assessment with three levels of "social learning" about uncertainties and relevant technical, governmental and societal aspects (van de Poel, 2017). These levels are 1) *impact learning*, which addresses uncertainties associated with the social impacts of a new technology, which can be both positive and negative; 2) *normative learning*, referring to what "we" think would be desirable or not and calls f or a balance between ensuring safety and being able to take some risk to gain knowledge of uncertainties; and 3) *institutional learning* addressing responsibility allocation, e.g., who decides what risk would be acceptable? And who establishes norms?

During the workshops, we made use of a case study that focuses on an emerging biotechnology application with several associated uncertainties and uncertain risks. Through this case and implementing the three levels of social learning, the discussions conducted in the workshops provided insights into tensions between the partaking stakeholder groups in terms of how to manage uncertainties and uncertain risks, what would be needed to overcome these tensions, and what would be needed to organize a learning process about these potential risks from emerging biotechnology applications? Based on these insights, we developed a tool–a script and guidelines–for researchers to organize a stakeholder workshop that enables a suitable environment in which learning processes can take place. Via this learning process, a range of partaking stakeholders can collectively identify different estimates of emerging risks and develop anticipatory strategies to lower or mitigate these. As a result, adaptations in (experimental) research designs can be defined to ensure safety, and knowledge gaps are identified for which complementary risk research should be set up.

2 Materials and methods

A total of five workshops were conducted; one in March 2021, two in June 2021, one in January 2022 and one in February 2022. Due to COVID-19, all workshops were conducted in an online environment with a maximum duration of 2.5 h. From all

TABLE 1 Script for Workshops conducted in March and June 2021. MOD, Moderator of the workshop; OBS, Observant (x3); "ConceptBoard" is an online platform which was used as an interactive discussion tool during these workshops.

Program part	Approx. Time	Content
Introduction	15	Welcome by MOD; Introduction of the workshop's program and room for questions; Introduction and more information regarding the case "Genetic Engineering and the Rhizosphere" by MOD.
Impact learning Identifying Possible Issues	30	*In breakout sessions* in *ConceptBoard*: What issues do the participants foresee based on the case? Can be both positive (opportunities) and negative (possible risks). MOD and OBS1, OBS2 and OBS3 help structure the identified issues by grouping them.
Break		
Normative Learning Prioritizing Issues	30	*In breakout sessions* in *ConceptBoard*: MOD and OBS 2 help the participants to provide argumentation concerning the importance of the identified values based on relevant values; Results in a group of associated values; In four rounds, participants are asked to prioritize the earlier identified values in terms of importance. To do so, participants have to explain why they feel that a certain value is more important than another? Every round, each participant can move one value one level up, and one value one level down. This results in an illustration of how each value is prioritized (low importance—moderate importance—high importance) *Plenary* discussion is devoted to the outcomes of the breakout sessions.
Institutional Learning	30	A *plenary* discussion devoted to the following questions: *How to balance (uncertain) risks and (potential) benefits?* *How to establish norms for uncertain risks?* *Who should be responsible to ensure safety?* *To what extent is the current risk management system able to cope with the identified issues?*
Evaluation and Closure	15	MOD asks all participants what their take-home message is; Thank you to all participants and request for feedback; Closure of workshop.

workshops, an anonymized transcript was made which was coded and analyzed accordingly. Prior to all workshops, participants signed a form giving consent to record the workshop (audio and video). Furthermore, of the five workshops, two (March and June 2021) were held in English, and three (June 2021, January 2022 and February 2022) were conducted in Dutch as all participants in these workshops were native Dutch-speaking. Therefore, quotes from these latter three workshops have been translated into English. All transcripts and original quotations are available upon request from the corresponding author[1].

2.1 Research design

There is a need for a constructive discussion about emerging risks and how to assess them/ learn about them responsibly. Using a case study, which is elaborated on in the next section, we first wanted to identify tensions between stakeholder groups that might complicate further communication and knowledge exchange between these groups. This mostly pertained to differing perspectives on emerging uncertainties and differences in the acceptability of these, possibly causing difficulty in progressing with experimental research safely and responsibly. All workshops were dedicated to gaining such insights.

As already mentioned, the workshops were built upon the pre-assessment step within the IRGC framework. But, to make this step more concrete for our workshop and to gain a more holistic approach, we have implemented the notion of social learning. Particularly its three levels of learning about uncertainties, namely: normative, impact and institutional (van de Poel, 2017). In the two workshops conducted in March and June 2021, a (plenary) discussion was devoted to each level of learning. Table 1 provides an overview of the organization of these workshops in the form of a short script. For the next "normative learning" step, we made use of an online discussion platform (i.e., ConceptBoard) that would make this step more interactive. Within the workshop, participants were divided into two "break-out" sessions and each was moderated either by MOD or by one of the present observers (OBS).

Based on derived insights from the workshops conducted in March and June 2021, we developed the first set-up of the tool to enable an environment suitable for discussing and learning about

1 https://doi.org/10.17026/dans-zta-6zz2

TABLE 2 Script for Workshops conducted in January and February 2022. MOD = Moderator of the workshop.

Program part	Approx. Time	Content
Introduction	30	Welcome by MOD;
		Introduction of the workshop's program and room for questions;
		Introduction and more information regarding the case "Genetic Engineering and the Rhizosphere" by MOD.
Identification and Prioritization of Risks	20	*In breakout sessions*: Participants identify and discuss possible issues they foresee based on the case. In these groups, they try to come to a top-3, in which the possible issues are listed in order of importance (e.g., dependent on estimated severity or magnitude).
	15	*Plenary*: Each 'break out group' presents their top-3. Each group is invited to pose questions to the other.
Break		
Formulating Anticipatory Strategies	15	*In breakout sessions*: Participants discuss and develop SbD strategies that might lower or mitigate the earlier identified issues. MOD stresses that these measures do not all have to be technically oriented, but can also focus on e.g. procedural or organizational matters.
	15	*Plenary*: Each "breakout group" presents their strategies and explains how these would mitigate or lower the earlier identified issues. Each group is invited to pose questions to the other.
Identify Needs of a Researcher	20	This part revolves around the question: *What do researchers need to implement (the earlier identified) SbD strategies in their research?*
		Participants are given 5 min to put things in the chat. Subsequently, each participant is allowed to elaborate on the matters they've put in the chat.
		Plenary discussion about what of the listed matters are found most important—can we reach a consensus?
Evaluation and Closure	15	MOD asks all participants about their take-home message;
		Thank you to all participants and request feedback;
		Closure of workshop.

uncertainties and uncertain risks. The workshops conducted in January and February 2022 were also dedicated to the validation of the tool, and therefore, these were slightly modified compared to the previous workshops. For instance, we decided to not use the interactive platform anymore as it turned out that participants were facing problems managing it in an online environment. Also, the workshops had more concrete steps which were: 1) identifying uncertainties and/or uncertain risks, 2) developing anticipatory strategies to lower or mitigate the earlier identified potential issues, and 3) determining what would be needed to implement the developed strategies in a researcher's experimental set-up. Step 2—developing anticipatory strategies–adheres to the notion of Safe-by-Design (SbD), a promising iterative risk management approach to deal with potential risks of biotechnology applications by using materials and process conditions that are less hazardous (Bollinger et al., 1996; Khan and Amyotte, 2003; Robaey, 2018). This choice was based on providing the partaking stakeholders with more concrete guidelines for developing suitable strategies, which also came up during the evaluation of the first two workshops. Table 2 provides a short script of these workshops.

All conducted workshops provided insights into tensions and/or differing perspectives between stakeholder groups about the identification of uncertainties and uncertain risks, and what would be needed to anticipate or mitigate these. In response, themes were derived that helped clarify and structure these insights, of which a detailed overview is provided in Section 3. Section 4 elaborates on the utilization of the developed tool and to what extent this format can be used to initiate an active discussion between stakeholders about uncertainties and uncertain risks associated with emerging biotechnology applications.

2.2 Case: Genetic engineering in the rhizosphere

As already mentioned, in 2017, Hogervorst and colleagues pointed out several developments in the biotechnology field for which, at that moment, no adequate environmental risk assessment could be conducted. One of these developments is the genetic engineering of plants' root exudates and their impact on the rhizosphere. The latter comprises the zone of soil around plants' roots that is influenced by root activity and consists of micro-organisms that feed on sloughed-off plant cells, proteins and sugars released by the roots; the root exudates (Walker et al., 2003). By manipulating a plant's root exudates, we can reduce our reliance on agrochemicals. Influencing the soil acidity in the plant root area can improve a plant's productivity (Bais et al., 2006; Ryan et al., 2009). For example, in papaya and tobacco plants, researchers have overexpressed the enzyme citrate synthase which is responsible for the production of citric acid in the plant. This acid is excreted through the roots of the plant

TABLE 3 Participants' Sectors and code.

Organization	
MOD	Moderator
OBS1	Observer
OBS2	Observer
OBS3	Observer/ Moderator
Workshop 16 March 2021	
RIT1	Research Institute—Technical Sciences
RIT2	Research Institute—Technical Sciences
RIT3	Research Institute—Technical Sciences
BSO1	Research Institute—Biosafety Officer
RIS1	Research Institute—Social Sciences
RO1	Regulatory Organization
RO2	Regulatory Organization
NG2	National Government
Workshop 3 June 2021	
RIT4	Research Institute—Technical Sciences
RIT5	Research Institute—Technical Sciences
RIS2	Research Institute—Social Sciences
RO3	Regulatory Organization
RO4	Regulatory Organization
Workshop 7 June 2021	
RIT6	Research Institute—Technical Sciences
RIT7	Research Institute—Technical Sciences
RIT8	Research Institute—Technical Sciences
RIS3	Research Institute—Social Sciences
RO5	Regulatory Organization
RO6	Regulatory Organization
Workshop 25 January 2022	
RIT9	Research Institute—Technical Sciences
RIT10	Research Institute—Technical Sciences
NG3	National Government
RO7	Regulatory Organization
BSO2	Research Institute—Biosafety Officer
RIS4	Research Institute—Social Sciences
Workshop 7 February 2022	
RIT11	Research Institute—Technical Sciences
RIT12	Research Institute—Technical Sciences
RIS5	Research Institute—Social Sciences
BSO3	Research Institute—Biosafety Officer
NG4	National Government
NG5	National Government
RO7	Regulatory Organization

and causes an acidifying effect on the plant's root zone. This effect can improve the availability of phosphate in the root zone, stimulating the plant's growth. Also, it can cause partial alleviation of aluminum toxicity stress, a frequently occurring problem in soils that inhibits plant growth (De La Fuente et al., 1997).

The rhizosphere is a complex environment with plants, microbes, soil and climate conditions interacting. As many of these interactions are not yet well understood, performing an adequate risk assessment is impossible at the moment. Therefore, such genetic engineering approaches have never progressed beyond experiments demonstrating the proof of principle. However, recently, scientists noted that they believe CRISPR-Cas9-based genetic screening can help future studies of plant-microbiome interactions and discover novel genes for biotechnological applications (Barakate and Stephens, 2016). Also, others argue that new tools and resources can be applied to introduce complex heterologous pathways–that encompass both natural and biosynthetic routes–into plants. Such would allow for building synthetic genome clusters from microbiomes to enable stacking and shuffling of disease resistance and stress tolerance traits between crop plants (Shih et al., 2016).

At the start of each workshop, the case described above was introduced to all participants, which illustrated the dilemma of having insufficient knowledge about such a complex system while it is also a technique that has potentially great societal benefits such as improving the global food supply. This set the stage for the workshop and formed the starting point for an active discussion on how to manage associated uncertainties and uncertain risks safely and responsibly.

2.3 Participants

As genetic engineering in the rhizosphere is a case with high complexity, many interactions between variables and insufficient knowledge on many aspects, a broad variety of stakeholders were invited to take part in this workshop–see Table 3. The aim hereby was to retrieve a holistic approach to uncertainties associated with the case and to develop a range of anticipatory strategies to lower or mitigate these uncertainties while taking into account both impact, moral and institutional aspects of risk management.

A total of 32 stakeholders from a range of expertise participated in the workshops. Participants' fields of expertise pertained to the technical sciences (i.e., microbiologists, biotechnologists, ecologists and Biosafety Officers), social sciences (i.e., (bio)ethicists, scholars working at the intersection of research and policy), regulatory organizations (i.e., risk assessors, policy officers) and the National Government (i.e., the Ministry responsible for national biotech regulation). We made sure that in every workshop a variety of stakeholders was partaking (see Table 3).

All participants were selected based on their knowledge of and/or experience with biotechnology applications and the regulation thereof. All hold senior positions in their

designated professions, except for participant [RIT3] who was an MSc. Student Biotechnology and [RIT1] and [RIT6] were both PhD Candidates at the time of the workshop. Also, [RIS1] and [RIT12] are both professor emeritus. Lastly, MOD, OBS1, OBS2 and OBS3 were present in all workshops.

3 Results

All discussions in the workshops were transcribed, coded and analyzed in line with the three levels of "social learning" (see Section 2 Materials and Methods). These levels formed the three themes that need to be addressed to arrive at responsible learning about uncertainties. These themes are 1) Institutional learning entailing responsibilities, 2) Impact learning considering uncertainties and uncertain risks, and 3) Normative learning adhering to balancing uncertain risks with potential benefits. Furthermore, as part of the workshops was devoted to developing anticipatory measures, the notion of SbD was also discussed. However, as SbD is not considered the main focus of this paper, insights from these discussions are integrated into the other themes. Sections 3.1–3.3 elaborate on the tensions and differing perspectives between stakeholder groups in line with the identified themes. Section 3.4 provides an evaluation of the conducted workshops and to what extent these have led to social learning, and a summary of the "lessons learned." These lessons functioned as input for the final design of the tool (i.e., the workshop script) which is elaborated in Section 4.

3.1 Institutional learning: Responsibility

The first identified theme revolves around responsibility concerning safety. With this, we refer to three matters; 1) researchers should apply a broad perspective on issues arising when developing a new technique or application thereof and taking anticipatory measures; 2) whether this should be done for both fundamental and applied research, and 3) unrealistic expectations concerning safety and the association with something being "natural" or not.

During the workshops, it became apparent that there is a consensus that researchers should make sure that their experiments are developed and conducted safely and responsibly. However, there were differences in how willing researchers would be to do so concerning possible long-term effects. On the one hand, participants [RIT9; RIS4; RO7] mentioned that researchers are probably not very willing to do so as they want to focus on answering the fundamental questions in research and generating new knowledge. In terms of long-term effects related to applications of their findings, stakeholders from other expertise might be better equipped to do so [RIT4].

"*The assumption here is somewhat that researchers want that too [talk and identify uncertainties], and I often find that very sobering when I speak to biotechnologists from [University], for example, who simply see that, that specific type of thinking is not their job at all. They mainly see the development of new knowledge as their task, and what risks there are is outsourced to, for example, [sub-department of University]. Or for a [regulatory organization] member.*" [RIS4]

"*I must also honestly say that I always try to keep myself a bit off from all the difficult follow-up things and think well, there are all [other] people who really like that and study bioethics, they can say useful things about it*" [RIT4]

Particularly in the light of the Asilomar Conference where researchers themselves took responsibility for ensuring the safe development of recombinant techniques (Berg et al., 1975; Berg and Singert, 1995; Abels, 2005), this was surprising. However, it was also argued that there certainly is a willingness amongst researchers, but tools need to be provided to do so [RIT10].

"*I do think that it is the researcher's responsibility to think about this [emerging risks or other use than intended], not just the university's. And I also think, on the one hand, there is some trust needed, that we [researchers] are certainly committed to... The whole purpose of the research we do is to make something better whether it's global health, the environment or whatever. So the benevolence is there. So, I need questions to be asked, for someone to point out a blind spot through a question, that makes me start to think about such. That's what I need!*" [RIT10]

In terms of these tools, discussions in the workshops of January and February 2022 were devoted to SbD strategies mitigating or limiting identified risks. Researchers would probably bear the most responsibility to "do" SbD as they are working with emerging techniques, but that would require to know when this should be done [RIT11], and to what it specifically pertains [NG4]. Would that only be when an application is already foreseen, or also during fundamental stages of research [RO7]?

"*It is important, when should you do this? And certainly if you are an academic researcher you have a fundamental question. And should you immediately start applying SbD because an application may result from your research? And when should you build in those reflection moments? And how do you build it in?*" [RIT11]

"*I make sure that I work safely, so [I] protect myself as a researcher and then I'm working [in a] SbD [way]. But that's not what we mean [with SbD]. But then you can say: when is it [SbD], and when is something not SbD? Does that mean you're always improving [on safety]? Or will there come a point where you say: look, we're here [it is safe enough]. Those are, I think, questions that are important for a researcher*" [NG4]

Some stakeholders pertaining to the social sciences domain argue that, from their perspective, researchers working on fundamental matters are not concerned with matters they consider outside their scope. For instance, [RIS4] argues that

when researchers are working on a fundamental matter, this would be value-neutral from their perspective, and therefore there is no need yet to consider whether this would be a good or bad idea. Only in the next steps e.g., when there is an envisioned application "we will look at what harm can it do?" [RIS4]. However, this was nuanced by a participant from the technical sciences [RIT7] who argues that there are two stages "We try to understand the world and then we try to change the world, to make our lives better". Thereby, [RIT7] acknowledges that applying insights one gained from *understanding* the world and trying to *modify* the world based on that knowledge are two different matters.

Also, there were discussions on what responsibility researchers have towards society in terms of communicating about risks and the meaning of safety–as biotechnology is still subject to public discourse. The discussion revealed two interpretations of safety, and how this is used and understood differently by different stakeholders. First of all, safety is often a technical matter in which a quantifiable chance of hazard (something that can cause harm) and how serious that harm could be is embedded–a definition that is frequently used by researchers from the technical sciences. However, the societal association with risks turns out to be ambiguous. Particularly in terms of risk communication, the societal interpretation of risk adheres more to the "absence of danger" [RO7; NG4]. In line with Beck et al. (1992), this association seems to be a response to society not being 'in control', but instead, organizations and governmental bodies responsible for the progress of biotechnological techniques and applications (Burgess et al., 2018). So, while technically safety refers to something having an acceptable risk involved, the societal interpretation is different.

"*Safety is also a concept defined by technicians, which is often where it comes from. And if we define safety as 'the chance is so small that something will happen' so we accept that, or we accept that because there is an advantage. But citizens understand safety as the absence of danger. So if you start talking about risks when you don't even know if they are there - they are always there of course—But, then you already have a negative communication frame. And at the same time, you cannot guarantee safety*" [RO7]

"*So we as a government think that we cover everything with [acceptable] risks, but in principle, the citizen says 'no, I want full protection'. Which of course is not realistic, you can never completely protect someone against something*" [NG4]

Also, there was some frustration detected in line with society's stance on biotechnologies. Not necessarily due to safety concerns–whether that would be having an acceptable risk involved or by being "fully protected from danger"—but due to the association made with naturalness (de Graeff et al., 2022). And, in that respect, when something is "natural" that it would be safe(r). [RIT4] mentions that the distinction between what is natural and what is not has become a bit blurry. It is mentioned that putting a UV lamp on crops is still natural as it is just ". . .putting the sun on it a little harder" and people tend to think very quickly that "natural is safe": "At least in the case when I talk to people about it, that's the biggest difference. If it's natural, then you can sell it. If it's not natural, alarm bells will start ringing." However, this association might be skewed as, given the recent pandemic, "corona is also natural and the vaccine we all receive is not natural" [RIT4].

3.2 Impact learning: Uncertainties and uncertain risks

Discussions also pertained to questions on appropriate strategies or measures to anticipate emerging risks, both short- and long-term. First of all, for short-term risks, strategies can be applied that limit possible risks. For example, one could ensure containment [RIT11; RIS5; RIT11; RIT1] or "that the plant is just one generation, or that you deprive the plant of the ability to reproduce" [NG4]. But for the long term, it might be a bit more difficult to understand issues arising and how to anticipate these properly. "So something is, typically in the lab, you will test something in the relatively short term, but we really rarely test for something in the long term. So there is lack of knowledge, usually for the long term effects" [RIT6]. On the other hand, participant [BSO3] mentions that taking heavy measures could be a strategy in itself to anticipate long-term risks. Lastly, [RIT11] questions how realistic this "testing for the long term" would be. In particular when a commercial party is involved: "How much time can you use to do this research? Especially if there is a commercial component to it. Ehm [sic], and that's why I think that long-term effects are especially difficult to capture in research, so to speak. You will not have 50 years to study those effects!" [RIT11]

Also, there was discussion about anticipatory strategies mostly being risk avoidant. Although that would be a way to ensure safe research, it doesn't solve the problem of learning about uncertain risks. Therefore, participants argued that there should be a distinction between strategies by which you aim to reduce uncertainties as much as possible, and strategies that make it possible to learn about the risks involved [RIS5]. However, some tension is expressed by stakeholders from the National government. On the one hand, although they prefer to choose the safest option from the start of a study, sometimes one does not know what the safest form is without researching it [NG4]. On the other hand, they [respective Ministry] are end-responsible for ensuring safety: "My role as a policy officer is to ensure that if something is genetically modified, it does not lead to a greater than a negligible risk to people, the environment and the living environment [sic]" [NG5] In that sense, learning about uncertain risks gives rise to a dilemma: ensuring safety and learning what the safest form is.

Following the discussion regarding strategies for avoiding risks and learning what uncertain risks entail, it was discussed

whether uncertainties should always be regarded as uncertain risks, and when uncertainties can be deemed a risk. It was mentioned that there can be a knowledge gap in such instances which can create tension in risk management. For example, for one material we know that we lack very specific information concerning the long-term toxicity levels in humans. While for another material, we might not even know yet whether or not this would be toxic to humans in the short term. This higher degree of uncertainty illustrates that there are varying degrees of missing information regarding uncertainties. But, this does not mean that all uncertainties should already be considered an uncertain risk (van Asselt and Vos, 2006, van Asselt and Vos, 2008; Flage and Aven, 2015). This is also addressed by participant [RO7]: "Look, if we don't know at all whether something has an effect, does it make sense to talk about risks? The fact that you say that there are risks, also means that you recognize that something is going on, and in this case, you don't know that at all!" [RO7].

Furthermore, it is mentioned that with uncertain risks we tend to focus on "known unknowns." However, given the vast pace of developments in the biotechnology field, it is expected that we will also have to deal with the "unknown unknowns" shortly–matters which we do not know yet. From a precautionary perspective, it would be justified to "keep our hand on the tap, and only open it when we know for sure what will come out!" [NG4]. Also, [RO2] mentions that as long you have insufficient data to obtain a proper view of the severity of risks, you should always assume the worst-case scenario. In other words: "if you don't have all data to be sure that something does *not* happen, you should assume that this *will* happen so the risk assessment generates that you should be more careful with taking the next steps (i.e., from lab to environment)" [RO2]. However, it is also argued that the best way to deal with these upcoming uncertainties is to work together and organize the systems in such a way that we are equipped to deal with new uncertainties:

"*In other words, you should set up the systems in such a way that if those [new] uncertainties arise, that you all [technical scientists, social scientists, regulatory organization, national government] know and trust each other enough to find solutions together. And which one [new uncertainty] you will encounter is indeed unknown, but at least then you have the structure to do something with it*" [RIS4]

"*Yes, so gather more brainpower from different perspectives to get a clear picture of what those new risks [of the new uncertainties] are*" [RO7]

In terms of working together, participants discussed examples coming from other disciplines where organizations are collaborating to learn about uncertainties. For instance, participant [RIS4] referred to a study once conducted about antibiotic resistance that could be possibly passed on by micro-organisms, and [RO7] to the "safe-innovation approach" in the field of nanotechnology.

"*There was one study about antibiotic resistance that could possibly be passed on by micro-organisms. This actually showed that a researcher could not come up with the question that the employee [a risk assessor from a regulatory organization] asked him, [presumably] based on his [the researcher's] own culture and knowledge and technological training. At the same time, that employee [from a regulatory organization] had no idea what was actually going on a fundamental, technical level of research. So in that [project's] user committee, the two of them seemed to really hit it off and thought: "yes, you have [combined] knowledge, we can only answer this question [thoroughly] together!*" [RIS4]

"*Yes, I'm thinking now, that comes from the 'nano-world' That's what they call* the safe-innovation approach. *I don't know if it's quite the same, but it is the commitment to . . . Let's say, the innovator and the people who have risk knowledge, to bring them together faster, so that you can have that conversation [about uncertainties]. And then it's just a question of whether those two are good enough, or whether you should include even more perspectives? So that's one of those thoughts that lives there and actually also in a protected environment, so to speak. So let's say 'Chatham House rules' or something. That you can just talk openly without company secrets just being exposed on the table, so to speak.*"[RO7]

Lastly, participants mention that using nature as a threshold could help to indicate whether an uncertainty should be regarded as an uncertain risk. "To know whether something involves a risk, you should also try to compare it to already known, you know, similar cases. [. . .] Also looking at what is already known about the type of changes that it might induce. And is that something that is already there in the environment?" [RO5]. However, discussions emerged about to what extent you could use nature as a reference, particularly if you are looking at a mechanism that is already present in nature, but that is also precisely the subject of intervention. "To what extent are they then [after intervention] comparable to mechanisms you find in natural systems?" [RIS5]. Also, how representative are tests performed under contained use? "For example, a soil in the greenhouse would already be tested there or say several soils: but how representative are they for the outside world, where it will eventually end up? It seems very complex to me to simulate a soil life and everything in the soil, so I think there is a modelling issue?" [NG3]. And, how desirable is it to mimic natural processes anyway? "Are natural processes always desirable and safe? So, is that always suitable to imitate? Nature has also developed enough dangerous situations and toxins, so what do we want to learn from nature and evolution?" [NG5].

3.3 Normative learning: Balancing risk and benefits

In all workshops, the potential benefits of developing technologies were mentioned and how these should be

balanced with uncertain risks. In particular for emerging technologies where there is a (societal) benefit associated, emphasis is often placed on not being able to guarantee that something is safe [RO2]. Gene drive technology is discussed–a technological application with possible great societal benefits by for instance altering or eradicating disease-causing insects such as mosquitos. For such technologies, society seems to be reluctant to accept possible associated risks even though the benefit would be large [RO1]. For (bio)medical applications (red biotechnology), this balance seems different which can be mostly explained by to who the risks and benefits are attributed [RO2; RO7; NG3].

"*For health care, this balance would be different as the benefits and risks would all be for the same person*" [RO2]

"*And whose benefits are they?" [RO7] "Yes, whose benefits and whose risks?*" [NG3]

"*Of course, it's about whose benefits and whose risks it is, isn't it? So if the risks are for society, but the benefits are only for the [producer], then you have a different story than if it were equally divided. Then you have a different weighting framework*" [NG3]

So, there appears to be a difference in how society perceives the risks associated with red biotechnology, and therefore, there might be less societal scrutiny for this strand of biotechnology. From a regulatory perspective, this strand is also regulated differently (Bauer, 2002, Bauer, 2005; Abels, 2005) and benefits (e.g., a life-saving treatment) are included in the respective risk assessment. For white (industrial) and green (plant) biotechnology, benefits are not taken into account during the risk assessment [RO2]. However, according to [RIS1], there is always a risk-benefit analysis performed, albeit implicitly. "One continues with these [research/experimental] activities because there are benefits. So, in every risk assessment, benefits are underlying because why would we proceed with them if there weren't any? So**,** implicitly there is always a risk/benefit weighing going on" [RIS1]. However, questions that emerged from this statement pertained to *who* makes, or should make, this (implicit) trade-off, and based on what information considering that the potential benefits are also uncertain. As justly mentioned by [NG3], "How should we account for these?" Following up on this remark, it was discussed that instead of trying to assign weight to the potential benefits and focusing on emerging risks, we could also look at what happens if we do nothing. For instance, related to the case, an expected benefit of engineering plants' rhizosphere is contributing to improving the global food supply: "What happens when we don't do it? Instead of well, just looking at what happens if we do it?", and "Perhaps exactly by intervening we can maintain an existing ecosystem, while otherwise, we would lose it, for example. So not intervening with nature can also lead to a loss of biodiversity and so on" [RIT4]. However, other participants were sceptical of the ideas introduced by [RIT4]:

"*And there is also seldom talked about the uncertainties in advantages, it is always said: we can do this and it all yields this nicely. I've never heard of any uncertainty about the benefits*" [NG3]

"*No, the premise is usually it's going to save the world. As long as the risks are manageable, we will save the world!*" [RIS4]

Also, if we would include potential benefits in the risk-benefit balance, and therefore proceed with these technologies, we might eventually be able to improve the global food supply. But, this could give rise to new problems–perhaps no direct risks to one's health, but more related to one's livelihood and quality of life, i.e., economic and financial independence.

"*Suppose this becomes the staple crop in some country that normally doesn't have such crops, say, will that displace other crops economically? Don't know if you understand what I'm saying, I'll give the example of Vanillin. You know, you can also do [produce] that with micro-organisms, but that means that in Madagascar suddenly less money is made with vanilla, and they suddenly have no income anymore. So that are other kinds of impact you could think about*" [RO7]

3.4 Evaluation and lessons learned

Based on the conducted workshops and the derived results presented in the previous sections, we first list the main findings and provide an evaluation of to what extent these workshops have contributed to social learning. Following upon, we formulate some "lessons learned" that formed the basis for the development of the final form of the tool to enable learning about uncertain risks which is presented in Section 4.

First of all, discussions associated with institutional learning entailed tensions about 3 matters: 1) responsibility allocation in the sense of researchers anticipating emerging risks, 2) whether these responsibilities should pertain to both short- and long-term risks and 3) apply to both fundamental and applied research. There was a consensus that ensuring safety is a responsibility that all associated stakeholders of an emerging technique or application should bear. In addition, researchers should be responsible to take anticipatory measures to lower or mitigate emerging risks, for instance through SbD. Based on this, some learning took place in the sense that participants are now aware of others' stance and perception on allocating responsibilities. However, as no consensus was reached in terms of what responsibility should be assigned to which actor, we can conclude that the conducted workshops have led to limited learning in terms of institutional learning.

Secondly, impact learning has taken place in the sense that emerging uncertain risks and uncertainties associated with the case study were identified. For instance; "I think that for me the take home message is that, indeed, that SbD is looked at very differently." And also: "When is something an uncertainty or a certain risk? And how should we as researchers deal with this?" [PIW], or: "So very often topics like this [the case study on engineering plants' rhizosphere] don't come up, but to participate in this discussion is certainly valuable. And if in the future, if these kinds of subjects become more topical for me,

it will help me a lot" [RIT9]. However, participants were not able to come to a consensus in terms of the possible impacts or severity of these uncertainties. This could be due to the different fields of expertise of the partaking stakeholders, e.g. some having less technical insight into the possible effects of the identified uncertainties. Furthermore, participants agreed that researchers should be equipped with tools to be able to anticipate 'new' uncertainties. For instance, different stakeholders working together and reorganizing the internal system as the external system (i.e., GMO regulation with a strong emphasis on the PP) currently provides little room to conduct research with uncertainties or uncertain risks involved–which also reflects institutional learning.

Lastly, normative learning took place during the workshop as the participants gained insights into the dilemmas accompanying emerging technologies, and balancing their pros and cons. Particularly concerning the latter, the participants had to weigh the pros and cons associated with the case study to list what potential risks they considered the most severe or probable, and how to anticipate these. However, learning in terms of establishing new norms or reshaping the process of ERA did not take place. There were suggestions made and discussions devoted to these matters but without concrete results. While this could be partly explained by the partaking stakeholders having little influence on these matters (i.e., EU-level decisions), it can also be attributed to how the current regulatory system is operationalized, in particular in terms of the PP. Although this principle could stimulate learning (i.e., specifically setting up risk research as a precautionary measure) which is argued by the European Commission (Commission of the European Communities, 2000), it now provides a very normative approach to risks in the risk assessment system. This has resulted in a system that allows learning about known risks (albeit limited as there is already extensive knowledge of these risks) but only very limited learning what uncertain risks entail–depending on the extent of knowledge that is missing. Research involving uncertainties, thus having very little to no knowledge about the extent, is limited as it cannot be proven to be safe, i.e. having acceptable risks.

The main findings of the conducted workshops formed the basis for the development of a workshop format that enables a constructive discussion about emerging risks with a broad range of stakeholders. First of all, we should focus on researchers and provide them with tools to create a mutual learning environment to identify and anticipate emerging risks, and set up research devoted to learning what uncertain risks entail. An important condition for this, however, is that such discussions take place in an informal, non-institutional setting. This way, a truly free exchange of views and perspectives can take place without shared insights immediately having implications in terms of (societal) perception or in terms of (stricter or less strict) regulation. Fear of such consequences or implications can result in stakeholders keeping information or opinions to themselves. Such issues have already emerged in the (conventional) chemical industry, where there is little incentive for industry to share knowledge and data about possible adverse effects (Drohmann and Hernandez, 2020; Bouchaut et al., 2022).

Secondly, in the workshop format, SbD should not be specifically mentioned as this notion is understood differently by stakeholders. This was mentioned in the workshops and is also argued in literature (Bouchaut and Asveld, 2020; Kallergi and Asveld, 2021). We want researchers to have an open vision to develop anticipatory strategies to lower or mitigate identified risks. If we would mention SbD specifically, this could lead to a "tunnel vision" in which strategies would only pertain to e.g. technical measures. Also, it is important that stakeholders have a shared vocabulary, or that it is accommodated that stakeholders elaborate on what they specifically mean with certain jargon. During the workshops, there were sometimes misunderstandings between stakeholders when using e.g., technical terms or jargon from the policy or regulatory domain. Although such misunderstandings were addressed, and partaking stakeholders that needed some explanation did ask for this, it does illustrate that stakeholders must feel comfortable with each other. While this is a challenge, we expect this to become more feasible once discussions of these matters have become more common. Also, referring back to "new" uncertainties emerging in the (near) future, making such constructive discussions common practice will be good preparation to be able to deal with these accordingly.

4 Enabling stakeholder communication

Based on the lessons drawn from the conducted workshops (Section 3.4), we developed a final workshop format intending to enable a constructive discussion about emerging uncertain risks and to develop anticipatory strategies for ensuring safety. To do so, we chose the format of a protocol that facilitates researchers to organize a stakeholder workshop themselves. First, we envision the workshop to be organized by researchers who are working with (emerging) biotechnologies or biotechnological applications and invite researchers from other relevant areas of expertise such as ecology and toxicology, as well as stakeholders from the regulatory regime and other scientific disciplines such as (bio) ethics, social sciences and Biosafety officers. Thereby, it's the intention that the organizing party composes a case of their own (as we have used genetic engineering of plants' rhizosphere). For instance, the development of a new type of application or proceeding from a laboratory environment (contained) to a non- or semi-contained environment (e.g., field trials or clinical trials) where new uncertainties or uncertain risks can emerge. Secondly, by organizing this workshop, insights are gained into; 1) different estimates of uncertain risks, which risks are identified, on what basis, degree and nature of

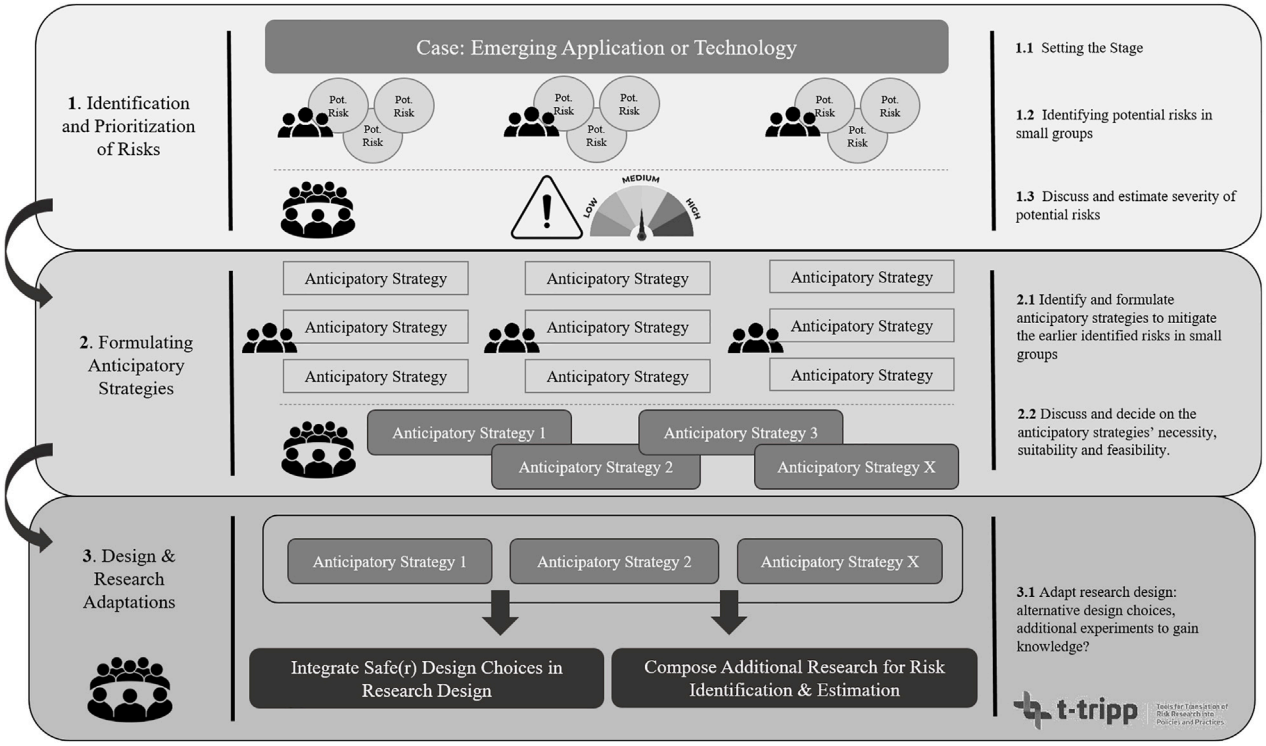


FIGURE 1
Flowchart for steps to follow during the social learning workshop.

uncertainty, 2) defining anticipatory strategies to mitigate or lower the identified uncertain risks, and 3) determining what is needed to implement the defined strategy/strategies in their research practices.

Also, during the workshops and based on the evaluation with all partaking stakeholders, it turned out that there needs to be some incentive for researchers to place more emphasis on the identification and anticipation of risks (both short- and long term). Therefore, we would like to stress that this workshop brings value to researchers by not only ensuring safe and responsible research design but a greater emphasis on identifying and anticipating uncertain risks could also speed up research later in the process. For instance, when an experiment is initiated, additional information on possible risks may be required by an organization's BioSafety Officer or a Member State's respective GMO Office. Having already invested in a more extensive analysis of emerging risks, such processes might be accelerated or even prevented. However, it can also occur that a risk assessment (e.g., at the start of a new experiment) reveals that the experiment involves uncertain risks and that more data or research would be needed. This would also be a moment to initiate a workshop that would complete the risk assessment more thoroughly. Also, consultation with an organization's BSO throughout the application process could create an incentive for organising this workshop. Therefore, we suggest researchers to organize this workshop when: researching emerging biotechnology applications; before composing or submitting a research proposal; when a risk assessment asks for extra information on emerging risks; and after consultation with an organization's BSO.

A detailed script to organize this workshop is provided in Supplementary Appendix A, listing all preparatory measures for the workshop, organizational and practical matters e.g. hosting the workshop online or in a physical setting, and the elaborate steps that need to be taken for the execution of the workshop. For instance, one or multiple moderators need to be appointed as the workshop largely consists of discussions. In addition, we have created a flowchart (Figure 1) that schematically illustrates the protocol and briefly lays out the three main steps that need to be followed during the workshop. This flowchart can also be used by the organization to keep an overview during the workshop. The first step in Figure 1 entails the identification and prioritization of risks. Here, after the case is introduced at the beginning of the workshop, participants identify and discuss potential risks in small groups. Following upon, a plenary discussion is devoted to each group's respective findings which are listed in terms of what potential risks are estimated the most important, which are considered less important, and why. In the second step,

participants again discuss in small groups what anticipatory strategies could be applied to lower or circumvent the identified risks in step 1. The groups then return to a plenary setting in which participants decide on what strategies are considered the most effective, efficient, or suitable considering the research set-up. The final step is a plenary discussion devoted to discussing what would be needed to implement the earlier developed anticipatory strategies and whether these would lead to an acceptably safe research design. If not, the participants identify the knowledge gaps and how these could be filled in by setting up additional (risk) research.

5 Discussion

In this paper, we presented the development of a tool, i.e. a script for researchers, to organize a workshop to identify emerging risks and anticipatory strategies associated with emerging biotechnologies utilizing the notion of social learning and its three levels of learning about uncertainties (i.e., impact, normative and institutional). Also, integrating notions associated with the SbD-approach provides researchers insights into adaptations concerning their research design for increased safety and setting up additional risk research specifically for learning about "new" risks. The following aspects deserve attention as they have an influence on the execution and the outcomes of the workshop: 1) stakeholder representation, 2) free knowledge exchange and actors in bad faith, 3) expertise in moderating, observing and reporting, 4) the choice of the case, 5) the use of definitions and jargon, and 6) some limitations of our proposed method.

First of all, stakeholder representation is crucial for obtaining a holistic overview of any potential issues arising, and the extent to lower or mitigate these. For example, when specific techniques or applications with a geographically broad focus are discussed, the participants must have the experience and knowledge to discuss the case study in such a broad context. The organizers of the workshop must be aware of and should not underestimate the needed diversity of participants in order to arrive at a constructive, inclusive and broad discussion. As this workshop is tailored to biotechnology research and developments, it makes sense to especially invite stakeholders who are associated with the technical aspects related to this field. However, evaluations after our conducted workshops revealed that also the presence of social scientists and policymakers is crucial to arrive at safe biotechnology development beyond technical aspects and measures, and was even greatly appreciated by the partaking stakeholders from the technical sciences. Considering the set-up of our workshop, the organizers will be from the technical sciences, who might not have stakeholders from the regulatory or societal domain in their direct network. Therefore, identifying and inviting these stakeholders might take up some considerable time and calls for extra preparations, which must be taken into account by the organizing party.

Following upon, having an informal setting is needed to arrive at "free" knowledge exchange in which stakeholders from differing domains exchange their thoughts and experiences, and can pose critical questions. This is particularly relevant when working with a controversial technique or application. Therefore, inviting a wide range of stakeholders, including both proponents and opponents, is crucial to arrive at applications that will not be rejected by society (von Schomberg, 2013). However, knowledge exchange can also be exploited by actors who will attempt to block every process that does not fit the direction they desire. This places organizers in a difficult position. Whose input is considered valuable, and who to exclude from the discussion? As this allows for selectivity, it also gives rise to another misuse of the knowledge exchange processes, namely that researchers can choose to only invite stakeholders who fit exactly with their research aims.

As discussion is a key element of the workshops, the organizers must have considerable expertise in moderating, observing and reporting. Although we provide the methods to organize a workshop, the organizers are responsible for the execution and thus the outcomes. Therefore, we recommend having a moderator with a neutral stance on the discussed technology or application. While it can be advantageous that the moderator is affiliated with the same lab that is developing the discussed technique (i.e. having specific technical knowledge), we do not recommend this as this may result in bias. This also applies to the observer(s) and reporter(s).

Usually, a case will be highly specific to a certain technique–as was the case used in our workshops. While this brings focus to the discussion, one should be careful about subsequently generalizing the outcomes of the discussions. Also, due to the high specificity of the case, it may be difficult for some stakeholders to grasp the content as it's not their field of expertise. On the other hand, the case being outside their "comfort zone" can also lead to obtaining new insights. Another issue concerns the timing of the introduction of the case to the participants. If already introduced before the workshop, the participants will be able to already think about the case and look up additional information. On the other hand, and also related to participants' own field of expertise, they may decline the invitation as they feel that this would be beyond their expertise, thereby risking that valuable new insights will be missed.

Discussions in our workshops also revealed that there was some confusion in terms of used jargon and stakeholders' definitions of e.g. uncertainty or risk, were not aligned. While having clear definitions is needed for effective communication, having differing definitions and interpretations can be used to shed light on stakeholders' different perceptions of notions related to risks and uncertainties–which could also be valuable for the organizing party.

Finally, organizers should be aware that the method we present here also has limitations. First of all, the case used for the conducted workshops pertained to a highly complex environment. Although this contributed to making the

dilemma clear of having insufficient knowledge, and continuing with promising developments, this may have caused some difficulties for participants to come up with concrete foreseen issues and anticipatory strategies. Secondly, in the case of the workshops we conducted, all stakeholders are associated with Dutch legislation. Although EU legislation is guiding, all EU Member States have their view on biotechnology and value different matters, and therefore, there might be a bias toward the Dutch perception. Thirdly, caution should be exercised when generalizing the outcomes of the workshop. Nevertheless, we believe that this tool is not only suitable to the field of emerging biotechnologies and can be used for other emerging fields such as nanotechnology or geo-engineering as well.

Author contributions

BB, HV and LA all contributed to the design and development of the workshops. BB performed the analysis on the conducted workshops, developed the tool–the flowchart and script–and wrote the first draft of the manuscript which was revised by HV and LA. All listed authors made an intellectual contribution to the work and approved it for publication.

Funding

This study was supported by the Netherlands Organization for Scientific Research (NWO) under grant number 15809 and is part of the research program "T-TRIPP: Tools for Translation of Risk research Into Policies and Practices."

Acknowledgments

The authors are grateful to Kyra Delsing of the Rathenau Insituut, The Hague, the Netherlands, for her help during the workshops, and the Institute's overall management of the T-TRIPP project. Furthermore, we want to thank all those who have participated in the workshops and for sharing their experience and insights with us.

Conflict of interest

HV is the owner of the company LIS Consult.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2022.946526/full#supplementary-material

References

Abels, G. (2005). The long and winding road from Asilomar to Brussels: Science, politics and the public in biotechnology regulation. *Sci. Cult. (Lond).* 14, 339–353. doi:10.1080/09505430500369087

Aven, T., and Renn, O. (2009). On risk defined as an event where the outcome is uncertain. *J. Risk Res.* 12, 1–11. doi:10.1080/13669870802488883

Bais, H. P., Weir, T. L., Perry, L. G., Gilroy, S., and Vivanco, J. M. (2006). The role of root exudates in rhizosphere interactions with plants and other organisms. *Annu. Rev. Plant Biol.* 57, 233–266. doi:10.1146/ANNUREV.ARPLANT.57.032905.105159

Barakate, A., and Stephens, J. (2016). An overview of crispr-based tools and their improvements: New opportunities in understanding plant-pathogen interactions for better crop protection. *Front. Plant Sci.* 7, 765. doi:10.3389/fpls.2016.00765

Bauer, M. W. (2002). Controversial medical and agri-food biotechnology: a cultivationanalysis. *Public Underst. Sci.* 11, 93–111. doi:10.1088/0963-6625/11/2/301

Bauer, M. W. (2005). Distinguishing red and green biotechnology: Cultivation effects of the elite press. *Int. J. Public Opin. Res.* 17, 63–89. doi:10.1093/IJPOR/EDH057

Beck, U., Lash, S., and Wynne, B. (1992). *Risk Society: Towards a new modernity*. California: Sage.

Berg, P., Baltimore, D., Brenner, S., Roblin, R. O., Singer, M. F., Bergt, P., et al. (1975). Summary statement of the asilomar conference on recombinant DNA molecules. *Proc. Natl. Acad. Sci. U. S. A.* 72, 1981–1984. doi:10.1073/pnas.72.6.1981

Berg, P., and Singert, M. F. (1995). The recombinant DNA controversy: Twenty years later. *Proc. Natl. Acad. Sci. U. S. A.* 92, 9011–9013. doi:10.1073/pnas.92.20.9011

Bollinger, R. E., Clark, D. G., Dowell, R. M., Webank, R. M., Hendershot, D. C., Kletz, T., et al. (1996). *Inherently safer chemical processes: a life cycle approach*. New York, NY: Center for Chemical Process Safety of the American Institute of Chemical Engineers.

Bouchaut, B., and Asveld, L. (2021). Responsible learning about risks arising from emerging biotechnologies. *Sci. Eng. Ethics* 27, 22. doi:10.1007/s11948-021-00300-1

Bouchaut, B., and Asveld, L. (2020). Safe-by-Design stakeholders' perceptions and expectations of how to deal with uncertain risks of emerging biotechnologies in the Netherlands. *Risk Anal.* 40, 1632–1644. doi:10.1111/risa.13501

Bouchaut, B., Hollmann, F., and Asveld, L. (2022). Differences in barriers for controlled learning about safety between biotechnology and chemistry. *Nat. Commun.* 13, 4103–4104. doi:10.1038/s41467-022-31870-8

Brisman, A. (2011). "Rio Declaration," in *Encyclopedia of Global Justice*. Editors D. K. Chatterjee (Dordrecht: Springer). doi:10.1007/978-1-4020-9160-5_648

Burgess, A., Wardman, J., and Mythen, G. (2018). Considering risk: Placing the work of ulrich Beck in context. *J. Risk Res.* 21, 1–5. doi:10.1080/13669877.2017.1383075

Commission of the European Communities (2000). Communication from the Commission on the precautionary principle. Brussels Available at: https://eur-lex.

europa.eu/LexUriServ/LexUriServ.do?uri=COM:2000:0001:FIN:EN:PDF [Accessed May 2, 2022].

de Graeff, N., Buijsen, M. B., and Bredenoord, A. (2022). On the nature of nature: A study on the use and meaning of nature and (un)naturalness in the literature on genetic modification. Available at: https://cogem.net/publicatie/on-the-nature-of-nature-a-study-on-the-use-and-meaning-of-nature-and-unnaturalness-in-the-literature-on-genetic-modification/ [Accessed May 2, 2022].

De La Fuente, J. M., Ramírez-Rodríguez, V., Cabrera-Ponce, J. L., and Herrera-Estrella, L. (1997). Aluminum tolerance in transgenic plants by alteration of citrate synthesis. *Science* 80276, 1566–1568. doi:10.1126/science.276.5318.1566

Drohmann, D., and Hernandez, F. (2020). Risk of regrettable substitution under EU REACH: Level playing field in the EU regulatory context. *ICRL - Int. Chem. Regul. Law Rev.* 2020. Available at: https://heinonline.org/HOL/Page?handle=hein.journals/icrl2020&id=30&div=&collection= (Accessed July 13, 2021).

Flage, R., and Aven, T. (2015). Emerging risk - conceptual definition and a relation to black swan type of events. *Reliab. Eng. Syst. Saf.* 144, 61–67. doi:10.1016/j.ress.2015.07.008

Hogervorst, P. A. M., van den Akker, H. C. M., Glandorf, D. C. M., Klaassen, P., van der Vlugt, C. J. B., and Westra, J. (2017). Beoordeling van risico's voor mens en milieu van nieuwe ontwikkelingen in de moderne biotechnologie. Available at: https://www.rivm.nl/bibliotheek/rapporten/2017-0178.html [Accessed March 17, 2022].

IRGC (2017). Introduction to the IRGC risk governance framework, revised version. *Lausanne EPFL Int. Risk Gov. Cent.*. doi:10.5075/epfl-irgc-233739

Kallergi, A., and Asveld, L. (2021). *Perceptions of safe-by-design for biotechnology*. doi:10.13140/RG.2.2.24999.62889

Khan, F. I., and Amyotte, P. R. (2003). How to make inherent safety practice a reality. *Can. J. Chem. Eng.* 81, 2–16. doi:10.1002/cjce.5450810101

Parisi, C., and Rodriguez Cerezo, E. (2021). *Current and future market applications of new genomic techniques*. Brussels: European Commision. doi:10.2760/02472

Renn, O., and Walker, K. (2008). in *Global risk governance. Concept and Practice using the IRGC framework*. Editors O. Renn and K. Walker (Germany: Springer). Available at: https://link.springer.com/content/pdf/10.1007/978-1-4020-6799-0.pdf (Accessed April 8, 2022).

Robaey, Z. (2018). *Dealing with risks of biotechnology : Understanding the potential of safe-by-design*. The Hague, Netherlands: Report commissioned by the Dutch Ministry of Infrastructure and Water Management. doi:10.13140/RG.2.2.13725.97769

Ryan, P. R., Dessaux, Y., Thomashow, L. S., Weller, D. M., Ryan, P. R., Dessaux, Y., et al. (2009). Rhizosphere engineering and management for sustainable agriculture. *Plant Soil* 3211, 363–383. doi:10.1007/S11104-009-0001-6

Shih, P. M., Vuu, K., Mansoori, N., Ayad, L., Louie, K. B., Bowen, B. P., et al. (2016). A robust gene-stacking method utilizing yeast assembly for plant synthetic biology. *Nat. Commun.* 71, 13215–13218. doi:10.1038/ncomms13215

van Asselt, M. B. A., and Vos, E. (2006). The precautionary principle and the uncertainty paradox. *J. Risk Res.* 9, 313–336. doi:10.1080/13669870500175063

van Asselt, M., and Vos, E. (2008). Wrestling with uncertain risks: EU regulation of GMOs and the uncertainty paradox. *J. Risk Res.* 11, 281–300. doi:10.1080/13669870801990806

van de Poel, I. (2017). "Society as a laboratory to experiment with new technologies," in *Embedding new technologies into society: A regulatory, ethical and societal perspective*. Editors D. M. Bowman, E. Stokes, and A. .Rip, 978–981.

von Schomberg, R. (2013). "A vision of responsible research and innovation," in In: R. Owen, M. Heintz, and J. Bessant (eds.) *Responsible innovation* (London: John Wiley), 51–74. Available at: http://onlinelibrary.wiley.com/doi/10.1002/9781118551424.ch3/summary.

Walker, T. S., Bais, H. P., Grotewold, E., and Vivanco, J. M. (2003). Root exudation and rhizosphere biology. *Plant Physiol.* 132, 44–51. doi:10.1104/PP.102.019661

frontiers | Frontiers in Bioengineering and Biotechnology

TYPE Original Research
PUBLISHED 07 October 2022
DOI 10.3389/fbioe.2022.979497

OPEN ACCESS

EDITED BY
Patricia Machado Bueno Fernandes,
Federal University of Espirito Santo,
Brazil

REVIEWED BY
Junjie Yue,
Beijing institute of Biotechnology, China
David Gillum,
Arizona State University, United States
Rebecca Mackelprang,
Engineering Biology Research
Consortium, United States

*CORRESPONDENCE
Craig M. Bartling,
bartlingc@battelle.org

SPECIALTY SECTION
This article was submitted to Biosafety and Biosecurity,
a section of the journal
Frontiers in Bioengineering and Biotechnology

RECEIVED 27 June 2022
ACCEPTED 31 August 2022
PUBLISHED 07 October 2022

CITATION
Gemler BT, Mukherjee C, Howland CA, Huk D, Shank Z, Harbo LJ, Tabbaa OP and Bartling CM (2022), Function-based classification of hazardous biological sequences: Demonstration of a new paradigm for biohazard assessments. *Front. Bioeng. Biotechnol.* 10:979497. doi: 10.3389/fbioe.2022.979497

Function-based classification of hazardous biological sequences: Demonstration of a new paradigm for biohazard assessments

Bryan T. Gemler, Chiranjit Mukherjee, Carrie A. Howland, Danielle Huk, Zachary Shank, Lela Johnson Harbo, Omar P. Tabbaa and Craig M. Bartling*

Battelle Memorial Institute, Columbus, OH, United States

Bioengineering applies analytical and engineering principles to identify functional biological building blocks for biotechnology applications. While these building blocks are leveraged to improve the human condition, the lack of simplistic, machine-readable definition of biohazards at the function level is creating a gap for biosafety practices. More specifically, traditional safety practices focus on the biohazards of known pathogens at the organism-level and may not accurately consider novel biodesigns with engineered functionalities at the genetic component-level. This gap is motivating the need for a paradigm shift from organism-centric procedures to function-centric biohazard identification and classification practices. To address this challenge, we present a novel methodology for classifying biohazards at the individual sequence level, which we then compiled to distinguish the biohazardous property of pathogenicity at the whole genome level. Our methodology is rooted in compilation of hazardous functions, defined as a set of sequences and associated metadata that describe coarse-level functions associated with pathogens (e.g., adherence, immune subversion). We demonstrate that the resulting database can be used to develop hazardous "fingerprints" based on the functional metadata categories. We verified that these hazardous functions are found at higher levels in pathogens compared to non-pathogens, and hierarchical clustering of the fingerprints can distinguish between these two groups. The methodology presented here defines the hazardous functions associated with bioengineering functional building blocks at the sequence level, which provide a foundational framework for classifying biological hazards at the organism level, thus leading to the improvement and standardization of current biosecurity and biosafety practices.

KEYWORDS

biohazard, sequence screening, virulence factor, biosecurity, biosafety

Introduction

The rapidly emerging discipline of bioengineering is enabling practitioners to analyze and assemble biological materials and microorganisms for industrial and research purposes through the creation of modified or novel organisms with specific functionalities (Slusarczyk et al., 2012). Bioengineering leverages sequences inspired from natural organisms that have been identified through studies in the life sciences (Figure 1). Exemplar chassis, such as *Escherichia coli* have been engineered with numerous functions, such as those to sense other bacteria, breakdown biofilms, and release toxic payloads (Hwang et al., 2017). While bioengineering is resulting in great benefit to mankind through medical advancements (e.g., precision medicine) and industrial use, the rapid progression and democratization of biotechnologies have presented new challenges for traditional biosafety and biosecurity practices.[1] Current biosafety practices often focus on organisms at the species level, instead of the functional level, which hinders the ability to predict and accurately prepare for previously uncharacterized organisms, such as biodesigns (i.e., engineered organisms) with novel functionalities. For example, focused by a selected list of pathogens, appropriate laboratory safeguards can be put in place using Biosafety Levels promoted by the Centers for Disease Control and Prevention (CDC), which are based on the severity of the disease and infectivity of the organism being manipulated (U.S. Department of Health and Human Services, 2014). While useful in the current paradigm, these biosafety practices do no enable objective and clear guidelines for engineered organisms outside of prioritized lists of species.

Beyond laboratory safety, frameworks to bolster biosafety practices are in place in some countries for research approval (US Department of Health and Human Services, 2017) and DNA ordering (US Department of Health and Human Services, 2022). Current DNA screening practices used by the International Gene Synthesis Consortium (IGSG) follow a uniform screening protocol against a Restricted Pathogen Database (RPD) "derived from international pathogen and toxin sequence databases" (International Gene Synthesis Consortium, 2017). While practical for regulated pathogens, screening sequences against the RPD has led to high false positive rates and requires time-consuming manual screening. In addition to hazardous pathogens and toxins, current best practices are in place for chemical synthesis and distribution of controlled drugs (U.S. Department of Justice. Drug Enforcement Agency. Diversion Control Division, 2019) and chemical weapons (Headquarters Department of the Army, 2018), but bioengineering is enabling bioproduction of such materials (e.g., (Galanie et al., 2015; Nakagawa et al., 2016)), which may also require extra precaution for laboratory manipulation. Given the exponential rise in DNA synthesis orders (Vickers and Small, 2018) and widespread creation of biodesigns, current screening practices using traditional approaches are unsustainable due to the high cost burden (due to high labor costs associated with reviewing sequences) relative to the increasing low cost of nucleotide synthesis. Thus, the need exists to shift from a subjective, organism-centric to an objective (and cost-effective), function-centric biohazard identification and classification system. This need is at the forefront of best practices as new draft guidance for screening synthetic nucleotide orders opens the aperture for screening to "sequences of concern" from select and non-select agents from all nucleotide sequence types—including short sequences (Federal Registar, 2022).

Here we introduce the term "hazardous function," which refers to one or more sequences (and associated metadata) that are associated with pathogenicity, toxicity, drug production, and other functions as described in this paper. Hazardous functions are driven by proteins that provide the organism or system (in the case of a cell free system or cell factory producing a toxin for example) with the necessary properties to cause infection or other detrimental effects. For example, lethal factor from *Bacillus anthracis* is a hazardous function, whereas DNA polymerase from *B. anthracis* is not. Manipulation of hazardous function sequences (e.g., recombinant production, genome insertion, mutation, etc.), even for legitimate purposes, could lead to the production of novel or enhanced hazardous products. In fact, precedent has shown that genetic manipulation can lead to biodesigns with high pathogenicity (van Der Most et al., 2000; Whitworth et al., 2005; Velmurugan et al., 2007; Bartra et al., 2008; Kurupati et al., 2010; Luo et al., 2010; Tsang et al., 2010), host bioregulation ability (Borzenkov et al., 1993; Borzenkov et al., 1994; Gold et al., 2007), vaccine escape capability (Serpinskii et al., 1996; Jackson et al., 2001; Zhang, 2003; Kerr et al., 2004; Chen et al., 2011), high transmissibility (Herfst et al., 2012), high toxicity (Francis et al., 2000), controlled drug production capability (Galanie et al., 2015; Nakagawa et al., 2016), and species extinction capability (Esvelt et al., 2014).

Hazardous functions identified through comparative genomic techniques (Gilmour et al., 2013) and related studies have been cataloged in databases containing virulence factors, toxins, and related other sequences (Supplementary Table S2). However, many of these databases are incomplete, poorly maintained, and/or do not have valuable metadata for objective biosafety assessments. Specifically, we and others have found that many of the entries in these databases simply tag sequences as "virulence factors" if attenuation of the activity

1 For this manuscript, the term biosafety refers to practices associated with protecting researchers from biological hazards associated with an organism based on its characteristics (e.g., practices associated with Biosafety Level 3 organisms). The term biosecurity refers the security of biological materials, including ordering of synthetic nucleotides. Thus, understanding the hazards associated with single synthetically made sequences can aid in biosecurity assessments (i.e., fulfilling synthetic nucleotide orders), whereas understanding the pathogenicity of an organism being manipulated in a laboratory can aid in biosafety assessments.

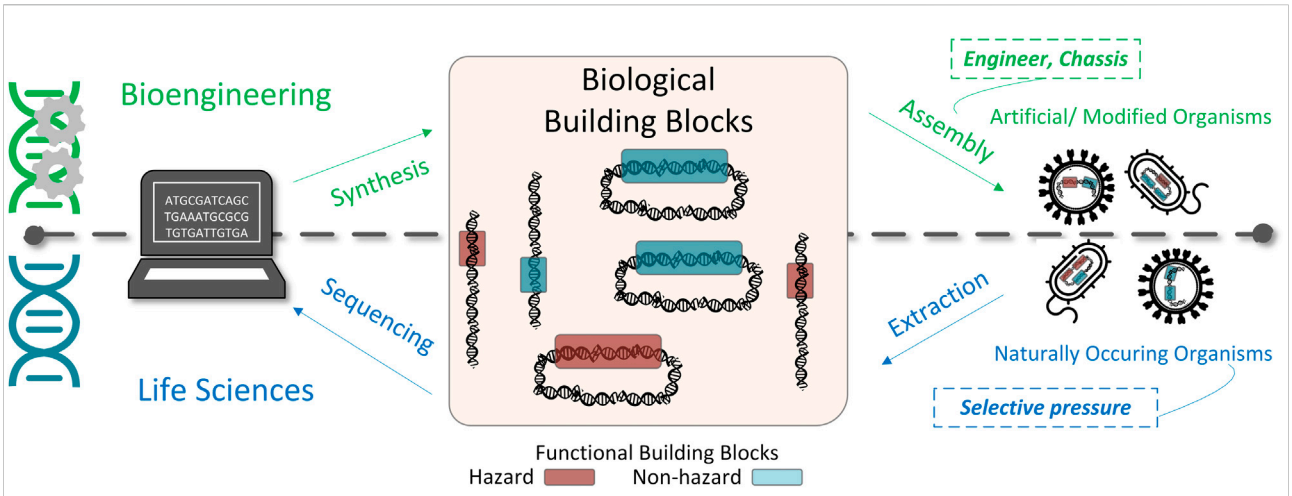


FIGURE 1
Understanding the hazard posed by bioengineering requires the characterization of hazardous building blocks. Bioengineering enables the creation of novel or modified organisms through oligonucleotide synthesis and assembly of building blocks within a biological vehicle. These biological functional building blocks are inspired from sequences extracted and sequenced from natural organisms. Such organisms can be composed of hazardous and non-hazardous functional elements (denoted by the red and blue blocks, respectively). The hazardous functions within organisms may have arisen from environmental selective pressures such as virus mutation to enable, for example, a jump from a vector to a human host, or a transfer of transposable elements among species.

leads to reduced virulence. Thus, many "virulence factors" may not be particularly hazardous in the context of bioengineering. For example, the Victor's Virulence Factors Database (Sayers et al., 2019) compiles bacterial virulence factors implied from published experimentation, such as large-scale mutational screens that seek to identify attenuated virulence phenotypes. Niu et al. illustrated the controversy associated with the term "virulence factor" by determining that 69% (1,368/1,988) of virulence factors in the Virulence Factor Database (VFDB) (Liu et al., 2019a) were common among pathogens and non-pathogens (Niu et al., 2013). In a more specific example, Segura et al. calls into question the definition of "critical virulence factors" for *Streptococcus suis*, suggesting that more scrutiny is needed before characterizing a strain as virulent based on clinical presentation, animal models testing, or *in vitro* tests (Segura et al., 2017). Taken together, current databases do not serve the purpose needed for biohazard identification necessitating the need for better definition and curation around hazardous functions. Godbold et al. recently described a controlled vocabulary called Functions of Sequences of Concern microbial pathogenesis research for bioinformatic applications (Godbold GD et al., 2021). Here we demonstrate the utility of these types of sequences of concern for understanding biohazards associated with bioengineering functional building blocks.

Regardless of the controversy associated with the term *virulence factor*, it is clear that different functions (and context) have different levels of importance for determining the sequence's overall hazard level and thus contribution to the organism or system's hazard level. Given such wealth of publicly available knowledge on the functions derived from genetic sequences in UniProt (and related databases), databases such as those presented in Supplementary Table S2, and the scientific literature at large, the scientific community is primed to enable function-based DNA sequence assessment to aid in the preparation for novel pathogens and/or components with hazardous properties as well as prevent nefarious development of novel engineered pathogens. To anticipate potential hazards associated with novel pathogens, Colf et al. called for "functionality-based approach" that focuses on key hazard elements such as stability of an organism, infectious dose, or toxicity (Colf, 2016), but such practices have not fully come to fruition. Here we introduce a paradigm of function-based sequence assessment that may fill the gaps associated with current biosafety practices. Hazardous functions can be subjective based on what the user considers a "hazard," but here we focus on functions associated with pathogenicity, toxicity, drug production, and other functions that can harm humans or other organisms of interest (e.g., livestock, crops, etc.). We first demonstrate our novel methodology to create a database of hazardous sequences classified into coarse functional categories. We then validate our methodology by demonstrating that a subset of the resulting database can be used to successfully distinguish pathogenic from nonpathogenic organisms via specific functional mechanisms. Finally, we further demonstrate the application of this methodology and resultant database through an example hazard scale. Therefore, the

methodology demonstrated here can immediately be used for biosecurity screening assessments of synthetic genes (through the exemplar hazard scale) and partial biosafety assessments for classification of bacterial pathogens and non-pathogens. Because our methods rely on the DNA sequence's encoded function, rather than agent-based lists, we provide a foundation for enabling function-based hazard assessments. This foundation can be built upon to provide comprehensive biosecurity and biosafety assessments for any novel biodesign through only analysis of the biodesign's genome.

Results

A methodology and database for function-based hazard assessments

To enable function-based biohazard screening, we developed an access-controlled biological Functional Hazards Database that contains protein sequences with metadata. The database documents sequences that have been verified in the laboratory to encode a hazardous function based on experimental information from the primary literature and/or publicly available databases (e.g., Supplementary Table S2). We have compiled these sequences and metadata into a machine-readable database that is focused on biohazards that target humans and non-humans of high economic value. Non-human hosts are based on an analysis performed by the United States Department of Agriculture Economic Research that demonstrated cattle, poultry, and swine comprised 96% of U.S. livestock farm receipts (of $176 billion) and corn, soybeans, and wheat comprised 48% of U.S. crop farm receipts (of 195.4 billion) (United States Department of Agriculture Economic Research Service, 2022) in 2017. Together, these six commodities comprise 71% of all U.S. farm receipts in 2017 (United States Department of Agriculture Economic Research Service, 2022).

We focus our database on particularly hazardous functions, which includes only a subset of virulence factor types as well as several hazardous functions not considered virulence factors (Figure 2). We delineate a virulence factor from a hazardous function as follows: while a virulence factor describes any factor (protein or otherwise) that aids in the virulence of organism, we define functional hazards as any sequence whose *verified* encoded *function* can lead to a direct and harmful impact on a host given a biological vehicle to do so. Thus, a logical division between hazardous functions and virulence factors (Figure 2) emerges based on this definition. Some traditional virulence factors are thus considered hazards, such as those involved in evading the host's immune system which–when encoded in an appropriate biological context (e.g., in *E. coli*)—contribute to direct detrimental impact to the host. In contrast, a transcription factor, for example, may only indirectly impact pathogenicity, and is thus not included in our hazard definition. We further delineate factors that are found throughout nature (i.e., those that are typically not unique to pathogens), such as siderophores, some secretion systems, and some non-protein virulence factor biosynthesis enzymes. For example, Type I and Type II secretion system proteins, which are ubiquitous throughout all gram-negative bacteria—pathogens and non-pathogens (Green and Mecsas, 2016)—are not considered hazardous functions in our definition. In contrast, Types III and IV secretion system proteins, which enable transport of potentially hazardous payloads across two gram-negative bacterial membranes and a host membrane, are considered hazardous functions. Further, careful consideration is given to particularly hazardous non-protein virulence factors such as endotoxin, which is biosynthesized by several enzymes (Raetz and Whitfield, 2002). More importantly, we consider several other sequence types that are not considered traditional virulence factors to be hazardous functions, such as prions, bioregulators, animal toxins (e.g., conotoxins), protein toxins (e.g., ricin), and proteins involved in the biosynthesis of small molecule toxins (e.g., saxitoxin) and drugs (e.g., morphine). For all hazardous sequences, we functionally classify the type of hazardous function into one or more of the 15 high level categories in Table 1 and elaborated below. These categories, chosen based on previous expert discussions from scientists with a variety of life science backgrounds, provide the basis for distinguishing pathogens and nonpathogens as shown by our validation and example biosafety assessment hazard scale discussed later.

Adherence, invasion, and motility

Adherence factors contained within our functional hazard database have experimental evidence (e.g., immunoprecipitation, cell binding assay, etc.) of a direct interaction with host membrane components. Interaction between the adherence factor and the host may enhance host cell tropism through direct interactions of a pathogenic apparatus that binds surface host cell receptors. Proteins that do not directly interact with the host but may be required for assembly of such a pathogenic apparatus can also be considered adherence factors but are further identified in our database as being dependent on direct adherence factors. For example, a type-4 pilus apparatus is responsible for adherence of *Neisseria meningitidis* to host receptors (Rudel et al., 1995), but is composed of several protein subunits. PilC and PilE have direct interactions with the host, whereas other proteins in the assembly do not (Bernard et al., 2014).

Invasion factors are those that leverage mechanisms such as Type III or Type IV Secretion Systems (T3SS/T4SS), pore formation, actin polymerization dysregulation, or cell lysis. The T3SS is a multi-protein needle complex that allows bacterial effectors to be delivered from the pathogen into the host cell directly. These effector proteins promote infection and suppress host defenses. For example, the *Yersinia pestis* T3SS

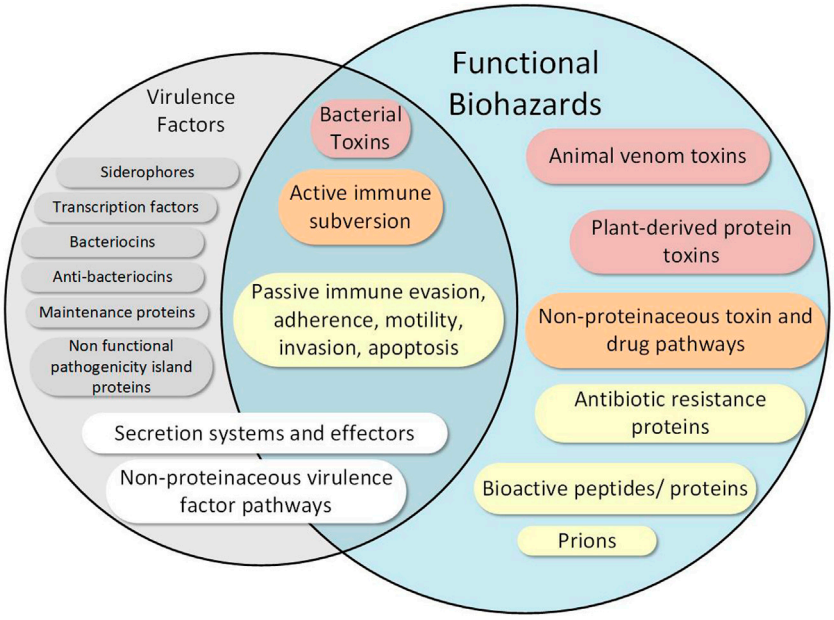


FIGURE 2
Functional biological hazards are differentiated from and include several functions beyond virulence factors. While some hazardous functions overlap with virulence factors, we define several hazardous functions outside the traditional definition of virulence factors. Many virulence factors that may be contained in avirulent organisms, such as siderophores and transcription factors, are not considered hazardous functions as they do not directly and uniquely perform hazardous functions. Hazardous functions are further described in the text and are color coded according coarse functional metadata groups as follows: Red—functions that do direct damage to cells such as toxins; Orange—functions involved in active host subversion or those involved in nonproteinacous toxin and drug pathways; Yellow—other virulence factors uniquely involved in pathogenicity (e.g., invasion), non-virulence factors that may contribute to detrimental host response (e.g., bioregulators and antibiotic resistance proteins), and prions; White—virulence factors that may also participate in non-hazardous microorganism functions; Gray—virulence factors that do not have a direct hazardous function. Note that the figure is non-exhaustive.

TABLE 1 Hazardous functional metadata categories.

Functional metadata	Definition
Adherence	Mediates pathogen or toxin binding to host cell
Motility	Enables a pathogen to move within or between host cells
Invasion	Enables a pathogen or toxin to actively enter or maintain protected spaces within the host
Inhibits host cell death	Inhibits host cell death
Host cell apoptosis	Leads to, aids in, and/or promotes host cell death
Passive host subversion	Passively works to avoid the immune surveillance, e.g., by altering recognizable elements of the pathogen
Active host subversion	Actively aggravates host immune detectors or effectors
Antibiotic resistance	Enables resistance of a pathogen to antibiotics
Damage	Actively damages host cells, host cell processes, or host barriers such as the extracellular matrix. Toxin sequences specifically contain the toxin activity gene ontology term (GO: GO:0009636)
Toxin pathway	Directly involved in the biosynthesis of a non-proteinaceous toxin
Drug pathway	Directly involved in the biosynthesis of a non-proteinaceous drug
Protein Bioregulators	Regulates cellular processes that can be detrimental to the host
Bioregulator pathway	Directly involved in the biosynthesis of a non-proteinaceous bioregulator that can be detrimental to the host
Prion	Protein that can misfold to become an infectious agent
Unknown	Hazardous function is unknown but contributes to complete or near complete loss of virulence when deleted or mutated

structure includes nearly 40 proteins (Cornelis, 2000; Frolkis et al., 2010). In *Y. pestis*, T3SS activation is triggered by cell contact and induces the secretion of effector proteins—termed Yersinia outer proteins (Yops)—across the host cell membrane where they inhibit bacterial phagocytosis and suppress the host immune response (Plano and Schesser, 2013). Like T3SSs, sequences such as bacterial pore-forming lysins and fungal cutinases, which can enable invasion through cleaving host cell walls (Sweigard et al., 1992; Dean et al., 2005; Chen et al., 2007; Basso et al., 2017) are included as well. Other types of invasive bacterial proteins, such as invasion plasmid antigen A (IpaA) from *Shigella sp*., which enables invasion through actin dysregulation (Izard et al., 2006; Park et al., 2011), are also included.

In addition to adherence and invasion, we include some motility factors, as some pathogens use mechanisms that allow a microbe to actively move between or within host cells following infection. This phenomenon is known as actin-based motility, which involves subversion of the host actin cytoskeleton to stimulate movement within the host cell, ultimately leading to microbial spread between cells. This rapid microbial dissemination is a critical step in many infectious diseases. For example, diseases caused by *Listeria monocytogenes* are caused in part by the protein ActA, which directly activates host actin polymerization machinery. This activation results in the formation of an actin "rocket tail" that propels the bacteria into adjacent cells, thereby infecting them (Finlay, 2005; Ireton, 2013).

Host cell death

During infection, pathogens work to maintain tight control of the host's intrinsic cell death mechanisms, often suppressing cell death then activating it to allow replication then dissemination, respectively. Induction of host cell death is used as a pathogenic strategy to allow a virus or bacteria to efficiently exit the host cell, spread to neighboring cells and access nutrients (Ashida et al., 2011). Further, by inducing host cell death, a pathogen can also eliminate immune cells and effectively evade immune defenses (Lamkanfi and Dixit, 2010; Ashida et al., 2011). Viruses are common proponents of this mechanism to facilitate dissemination of replicated virus and suppression of the immune system. For example, the human immunodeficiency virus (HIV), induces programed cell death in healthy T lymphocytes, contributing to the gradual T cell decline and ultimately acquired immune deficiency syndrome (Ahr et al., 2004; Romani and Engelbrecht, 2009). Thus, proteins such those that promote this induction of apoptotic signal (Vpr and HIV envelope proteins) are including in our database (Ayyavoo et al., 1997; Ahr et al., 2004; Romani and Engelbrecht, 2009). In contrast to induction of host cell death, inhibition of host cell death is also a hazardous function since host cell death can be used as an immune defense mechanism to contain the spread of the infection. These hazardous functions enable a pathogen to promote its overall survival within the host by giving the pathogen more time to colonize efficiently prior to dissemination. Enteropathogenic *Escherichia coli*, for example, uses this strategy to stall premature host cell death during infection through the EspZ effector protein, which activates pro-survival signaling pathways within the host (Shames et al., 2010; Shames and Finlay, 2010).

Passive and active host subversion

Pathogens can also evade the host by avoiding or aggregating more specific host immune defenses than those discussed above. Microbes have evolved numerous and diverse strategies to circumvent the host immune system, many even using multiple mechanisms. We classify these strategies as passive or active, in which hazardous functions act to either avoid host immune surveillance or actively interfere with the host's immune responses, respectively. Common passive mechanisms include using antigenic variation, epitope masking, and the use of decoys or molecular mimicry. Often, circumvention of host detection is accomplished by a virulence factor altering recognizable elements of the pathogen. For example, Ebola virus glycoprotein (GP), a key antigen in Ebola pathogenesis, can evade host immune defenses by epitope masking and steric shielding (Cook and Lee, 2013; Wong et al., 2014). Steric shielding of surface epitopes by glycans also prevents antibody binding and binding of host major histone compatible complex I and β1 integrins with other immune cells, thereby preventing the host immune response (Francica et al., 2010). Ebola virus also leverages decoy mechanisms by producing large quantities of secreted GP proteins that adsorb host antibodies (Blair et al., 2015).

In contrast to passive subversion, active host subversion involves active interference with the host's immune responses. For such interference, a microbe must produce factors that are able to block or modulate specific steps in the immune response cascade (Schmid-Hempel, 2009). These factors can be membrane-bound or directly injected directly into the host cell using secretion systems such T3SSs, as discussed above (Raymond et al., 2013). Many bacteria possess efficient means of evading the host complement system. For example, chemotaxis inhibitory protein (CHIPS) from *S. aureus* can bind receptors on neutrophils, blocking their recruitment and engagement to resist complement-mediated killing (Rooijakkers et al., 2005). Active evasion of the immune system can also be accomplished by interfering with the immune response signaling network. For example, *Yersinia* Yop proteins downregulate the expression of TNF-α, thereby effectively blocking pro-inflammatory signaling (Sweet et al., 2007; Schmid-Hempel, 2009).

Antibiotic resistance

Just as pathogens can evade endogenous host responses, pathogens have evolved to evade exogenous factors, such as antibiotics, through expressing hazardous functions.

Surveillance of these hazardous functions is critical, as the rapid and broad dissemination of antibiotic resistance determinants by lateral gene transfer has been demonstrated throughout diverse bacterial species. Several mechanisms have been described that can lead to antibiotic resistance including: production of enzymes capable of metabolizing or modifying antibiotics, antibiotic binding-site modifications to prevent binding, production of outer membrane components that confer low permeability, and overexpression of multi-drug efflux pumps (Fournier et al., 2006; Vila et al., 2007; Kempf and Rolain, 2012; Blair et al., 2015; Bakour et al., 2016; Geisinger and Isberg, 2017). Bacteria often employ more than one mechanism of antibiotic resistance, leading to multidrug-resistant strains. For example, methicillin resistant *S. aureus* (MRSA), produce both β-lactamases that can inactivate β-lactam antibiotics (e.g., penicillin), as well as proteins acquired by lateral gene transfer (PBP2a proteins) that confer resistance to methicillin (Chambers, 1997; Stapleton and Taylor, 2002). While antibiotic resistance factors can be hazardous, the context of the factors needs to be carefully considered. Often antibiotic resistance has been shown to result in virulence attenuation (Andersson and Hughes, 2010; Geisinger and Isberg, 2017), but some studies demonstrate that resistance has increased pathogenic potential during infection (Luo et al., 2005; Skurnik et al., 2013; Roux et al., 2015). While the precise correlation between virulence and antibiotic resistance remains unclear, we define antibiotic resistance as hazardous function given reasonable context (i.e., contained within a pathogen).

Damage

Perhaps the most hazardous functional category can be considered one that does direct damage to the host. While some of the above hazardous functions can directly damage the host, biological toxins represent the largest class of directly damaging hazardous functions. According to the Gene Ontology Consortium, biological toxin activity involves the selective interaction "with one or more biological molecules in another organism (the "target" organism), initiating pathogenesis (leading to an abnormal, generally detrimental state) in the target organism" (EMBL-EBI, 2019). Biological toxins may be proteinaceous or non-proteinaceous, with protein toxins often consisting of multiple subunits that attribute to virulent functions for adherence, invasion, and inactivation of critical cellular functions. Toxins are highly diverse, even within some toxin types. For example, possibly hundreds of thousands of conotoxins—antagonists or agonists of various receptors and ion channels—exist (Lewis et al., 2012). Examples of proteins relevant to this category included in our hazardous function database are shown in Supplementary Table S4.

Pathways

In addition to protein toxins, our database includes key enzymes involved in the biosynthesis of fully and partially characterized small molecule toxin pathways, such as those that produce aflatoxins (cancer-causing and cellular process-disruption fungal toxins (Haschek and Voss, 2013; National Cancer Institute, 2019)), trichothecenes mycotoxins (protein synthesis-inhibiting fungal toxins (Kiessling, 1986)), microcystins (cyanobacterial serine/threonine protein phosphatase-hepatotoxins (Tillett et al., 2000; Campos and Vasconcelos, 2010)), tetrodotoxins (bacterial sodium channel-blocking neurotoxins) (Jal and Khora, 2015; Lago et al., 2015; Magarlamov et al., 2017), and saxitoxins (bacterial sodium channel-blocking neurotoxins) (Al-Tebrineh et al., 2010).

Beyond hazardous pathogens and toxins, we also consider naturally derived or inspired drugs. Bioengineering is presenting a new challenge to control the production of these naturally derived drugs, as the starting materials may not be regulated. Some drugs, such as opiates and cannabinoids, are produced naturally in plants, and have been demonstrated to be produced in yeast and bacteria (Galanie et al., 2015; Poulos and Farnia, 2015; Nakagawa et al., 2016). Illicit drugs pose a hazard to public health and the economy and are thus controlled by the US Drug Enforcement Administration (DEA) using a five category classification system (United States Drug Enforcement Administration, 2019), with schedule I drugs being the highest hazards as they have no currently accepted medical use and have a high potential for abuse (e.g., heroin and cannabis). For chemical synthesis, supplies to synthesize drugs are regulated by the US government (U.S. Department of Justice. Drug Enforcement Agency. Diversion Control Division, 2019), but biosynthetic supplies are less regulated and may thus present a gap in biosecurity and biosafety. Our functional hazards database thus includes exemplar pathways such as the opioid and cannabinoid pathways, which are fairly well elucidated (Galanie et al., 2015; Nakagawa et al., 2016) as well as sequences from less characterized pathways, such as the cocaine pathway (Jirschitzka et al., 2012).

Bioregulators

We also consider host regulators as well, since such molecules can ultimately lead to manifestations of disease (Goldman, 2000) and have drug-like activity. These bioregulators can be peptides, proteins, and small molecules produced naturally by the host in response to an insult or produced by other organisms (e.g., amphibians). Further, regulatory peptides have been discovered and created to mimic small molecule regulators such as opioids (Dudak et al., 2011; Aldrich and McLaughlin, 2012). Like antibiotic resistance factors, the context and scope of bioregulators must be carefully considered. While many bioregulators can be considered hazardous, we limited our initial database to those that could have a high impact on human systems such as the cardiovascular, nervous, and immune systems (Supplementary Table S3).

Prions

Prions are considered a functional hazard as well. A prion is a protein that can misfold to become an infectious agent

(i.e., transmitted from one host to another). Prions most abundantly occur in the brain and are responsible for a variety of fatal progressive neurodegenerative disorders called transmissible spongiform encephalopathies (Prusiner, 1998). The causative agents of these diseases are normal cellular prion proteins (PrPC) that have undergone a posttranslational conformational change into an abnormal scrapie prion protein (PrPSc) (Huang et al., 2015). PrPSc proteins are able to transmit the pathological conformation to PrPC through poorly understood mechanisms (Dobson, 2001; Huang et al., 2015; Erana and Castilla, 2016). Notable prions included in our database are those that lead to Bovine Spongiform Encephalopothy (BSE, or "mad cow disease"), Creutzfeldt-Jakob disease in humans, feline spongiform encephalopathy in cats, and exotic ungulate encephalopathy in zoo animals (Wells et al., 1987; Wilesmith, 1994; Will et al., 1996). Although these diseases are rare, they are usually rapidly progressive and fatal and synthetic versions can induce pathology in experimental animals (Telling et al., 1995; Legname et al., 2004).

Unknown

While many hazardous functions have distinct mechanisms, we do consider potentially hazardous functions with nonspecific mechanisms as well. Throughout the database compilation process, we identified several instances where a protein sequence likely contributes to a hazardous function, but the exact mechanism is unknown. For example, our database contains a relatively high number of Mycobacterium sequences since we leveraged many of the virulence factors documented in PATRIC (Wattam et al., 2017), which relied mainly on one study. In this study, the authors identified which genes are required for *in vivo* growth (and not *in vitro* growth) (Sassetti and Rubin, 2003). Thus, while many of these genes are considered to potentially contribute to hazardous functions, their actual functions are unknown.

Validation of the methodology and resulting functional hazard database: Identification of hazardous functions

To validate our methodology of identifying, categorizing, and databasing hazardous sequences, we leveraged the studies presented in Table 3, which segregate various pathogenic and nonpathogenic bacterial species. We identified eight different organism groups and separated species in each group into pathogens and nonpathogens. We further categorize the pathogens into species and/or disease-causing groups. With the exception of *Pseudomonas syringae* (a plant pathogen), all species leveraged in this validation are pathogenic to humans and/or economically critical livestock. For the validation, we aligned the coding sequences (CDSs) from each strain against a subset of our database that contained only hazardous function sequences from each of the eight organism groups. We used a subset of our database to reduce potential noise associated with hazardous functions potentially encoded in nonpathogens as a proof of concept for the method; thus any use of this methodology for biosafety assessments should note this limitation. We scored each CDS alignment hit as the *(percent identity)* × *(percent hazardous sequence coverage)* and normalized each hit to the total number of CDSs contained in the strain. The normalization step was performed since, for example in the case of *E. coli*, 1 Mb genome size differences can occur among strains, leading to different pathotypes (Dobrindt, 2005). To count the fraction of hazardous CDSs in each strain, we considered different alignment thresholds to ensure that a specific alignment cutoff did not impact our results. Specifically, the fraction of hazardous sequences is nearly unchanged between 20 and 80% alignment scores for all groups (data not shown). Importantly, the fraction of hazardous functions in pathogenic species compared to nonpathogenic species is higher across the entire range in nearly all cases.

Table 2 shows the number and percentage of hazardous CDSs using a relatively stringent alignment threshold of 40%. The 40% threshold has previously been demonstrated to be a useful cutoff by Suzek et al. (2015). In the referenced study, the authors showed 97% of Uniref50 cluster members, defined by the 40% threshold (≥50% sequence identity over 80% sequence coverage (UniProt, 2019a)), share identical or similar gene ontology terms (i.e., have the same function) (Suzek et al., 2015). Thus, this threshold is useful for CDSs that have identical or similar functions relative to sequences contained in the hazardous function database. Table 3 outlines that the average number and fraction of CDSs identified for each pathogenic and nonpathogenic group using the 40% threshold. In 19 out of 21 pathogenic groups, the percentage of CDSs is higher for pathogens compared to nonpathogens (16/18 being significantly higher), suggesting that our methodology was successful in identifying hazardous functions for these groups.

We further identified specific hazardous functions enriched in each pathogenic group (Supplementary Table S1). For this analysis, we assumed (based on testing, data not shown) that a function is "enriched" in a pathogenic group compared to its nonpathogenic counterpart if the average alignment score across all strains in the group is ≥60% higher than the average in the nonpathogen group or the average in the nonpathogen group is 0% and the average in the pathogen group is ≥40%. As a control, we also determined if any hazardous functions are enriched in the nonpathogen group (i.e., if the average alignment score in the nonpathogenic group is ≥60% higher than the pathogen group or the average in the pathogen group is 0% and the average in the nonpathogen group is ≥40%). Based on this analysis, we identified 379 total enriched functions in the pathogenic groups compared to only 12 total hazardous functions in the nonpathogen groups. The pathogen groups averaged 19 enriched hazardous functions across the various pathogen groups (range 1–70, Supplementary Table S1). These functions were involved in a variety of

TABLE 2 The average number and percentage of hazardous CDSs are greater in pathogenic groups compared to nonpathogenic Groups.

Organism	Group	Genera/species in group	Average ± SD of # CDSs with hazardous functions (Average % CDSs)[a]
Neisseria	Pathogenic	*N. meningitidis*	***50 ± 3 (2.3%)***
		N. gonorrhoeae	***53 ± 3 (2.2%)***
	Nonpathogenic	See Table 3	27 ± 5 (1.3%)
Escherichia coli	Pathogenic	EAEC/ETEC/AIEC/EPEC	160 ± 31 (3.2%)
		EHEC	***290 ± 20 (5.3%)***
		ExPEC	***163 ± 32 (3.3%)***
	Nonpathogenic	See Table 3	125 ± 26 (2.7%)
Burkholderia	Pathogenic	*B. mallei*	***111 ± 17 (2.1%)***
		B. pseudomallei	***143 ± 16 (2.1%)***
		B. cenocepacia	***102 ± 8 (1.5%)***
	Nonpathogenic	See Table 3	82 ± 20 (1.2%)
Pseudomonas	Pathogenic	*P. aeruginosa* and *P. mendocina*	***126 ± 21 (2.3%)***
		P. syringae (plant pathogen)	76 ± 3 (1.3%)
	Nonpathogenic	See Table 3	76 ± 7 (1.9%)
Streptococcus	Pathogenic	*S. pneumoniae*	***39 ± 6 (1.9%)***
		S. pyogenes	***42 ± 3 (2.2%)***
		S. suis	***33 ± 4 (1.6%)***
	Nonpathogenic	See Table 3	20 ± 2 (1.0%)
Bacillus	Pathogenic	*B. cereus* and others (See Table 3)	***59 ± 11 (1.1%)***
		B. anthracis	***61 ± 3 (1.1%)***
	Nonpathogenic	See Table 3	23 ± 10 (0.5%)
Clostridium	Pathogenic	*C. botulinum* and *C. tetani*	***6 ± 1 (0.3%)***
		C. difficile	5 ± 1 (0.2%)
		C. perfringens	***5 ± 1 (0.4%)***
	Nonpathogenic	See Table 3	1 ± 1 (0.1%)
Mycobacterium	Pathogenic	*M. tuberculosis* and others (See Table 3)	***440 ± 8 (26%)***
		M. leprae and others (See Table 3)	***281 ± 120 (18%)***
	Nonpathogenic	See Table 3	288 ± 19 (12%)

[a]CDSs above the 40% threshold as defined in the Methods Section; the fraction of CDSs is defined by the number of hits divided by the total number of CDSs in each strain.
Bold italics represents a significant difference in percentage between the pathogenic and nonpathogenic group as defined by a pairwise *t*-test ($p < 0.05$, two-tailed, unequal variance).

processes such as adherence, immune evasion, antibiotic resistance and damage (including toxin activity). The hazardous functions identified to be enriched in the nonpathogen groups mapped to four functions in the *E. coli* group (required for colonization but with unknown mechanisms), one antibiotic resitance function in the *P. syringae* group, three functions in the in the *S. pyogenes* group (involved in antiphagocytosis but with unknown mechanisms), and four antibiotic resistance functions in the Mycobacterium groups. Thus, the results in Supplementary Table S1 suggest that our database enables successful identification of enriched hazardous functions from pathogens as compared to their nonpathogenic counterparts.

TABLE 3 Genomic data from pathogenic and nonpathogenic strains used in this study.

Type	Genera/ Species organism group	References	Pathogenic groups: species/strains (#)	Nonpathogenic groups: species/strains (#)	# Hazardous functions in database
Gram-negative bacteria	Neisseria	Lu et al. (2019)	*1. N. meningitidis* (85) *2. N. gonorrhoeae* (15)	*N. lactamica* (3); *N. longa* (1); *N. zoodegmatis* (1); *N. longate* (1)	67
Gram-negative bacteria	*Escherichia coli*	Cosentino et al. (2013)	1. EAEC/ETEC/AIEC/EPEC (11) 2. EHEC (8) 3. ExPEC (10)	K-12 (2); other non-pathogenic strains (13)	374
Gram-negative bacteria	Burkholderia	Cosentino et al. (2013)	*1. B. mallei* (4) *2. B. pseudomallei* (4) *3. B. cenocepacia* (4)	*B.* sp. *CCGE1001* (1); *B.* sp. *YI23* (1); *B. glumae BGR1* (1); B. *phymatum STM815* (1); *B. phytofirmans PsJN* (1)	141
Gram-negative bacteria	Pseudomonas	Cosentino et al. (2013)	*1. P. aeruginosa* (5) and *P. mendocina* (2) *2. P. syringae* (3)	*P. brassicacearum* (1); *P. fluorescens* (2); *P. putida* (6); *P. stutzeri* (1)	175
Gram-positive bacteria	Streptococcus	Cosentino et al. (2013)	*1. S. pneumoniae* (9) *2. S. pyogenes* (13) *3. S. suis* (9)	*S. parauberis* (1); *S. salivarius* (3); *S. thermophilus* (5)	161
Gram-positive bacteria	Bacillus	Cosentino et al. (2013)	*1. B. cereus* (6); *B. cytotoxicus* (1); *B. weihenstephanensis* (1) *2. B. anthracis* (5)	*B. amyloliquefaciens* (4); *B. atrophaeus* (1); *B. cellulosilyticus* (1); *B. cereus Q1* (1); *B. clausii* (1); *B. coagulans* (2); *B. halodurans* (1); *B. megaterium* (1) *B. pumilus* (1); *B. selenitireducens* (1); *B. subtilis* (4)	116
Gram-positive bacteria	Clostridium	Cosentino et al. (2013)	*1. C. botulinum* (8) and *C. tetani* (1) *2. C. difficile* (2) *3. C. perfringens* (3)	*C. acetobutylicum* (3); *C. beijerinckii* (1); *C. cellulovorans* (1); *C. clariflavum* (1); *C. kluyveri* (2); *C. lentocellum* (1); *C. ljungdahlii* (1); *C. phytofermentans* (1); *C. saccharolyticum* (1); *C.* sp. *SY8519* (1); *C. thermocellum* (1)	54
Bacteria	Mycobacterium	Andreevskaia et al. (2006); Cosentino et al. (2013); Ilina et al. (2013); Prasanna and Mehra (2013)	*1. M. africanum* (1); *M. avium* (1); *M. bovis* (1); *M. canettii* (1); *M. tuberculosis* (5) *2. M. abscessus* (1); *M. avium* (1); *M. leprae* (2); *M. marinum* (1); *M. ulcerans* (1)	*M.* sp. *KMS* (1); *M. gilvum* (1) *M. rhodesiae* (1); *M. smegmatis* (1); *M.* sp. *JLS* (1); *M.* sp. *MCS* (1) *M.* sp. *Spyr1* (1); *M. vanbaalenii* (1)	339

Validation of the methodology and resulting functional hazard database: Hazard fingerprints

To validate the classification component of our methodology (Table 1), we leveraged our functional categories to create "hazard fingerprints" for each strain. The fingerprints were calculated by summing the alignment scores for the CDSs for each strain that belong to each functional category. For these alignments, we accounted for both highly confident hazardous CDSs (e.g., those with alignment scores >40% to our database) as well as less confident, yet potentially hazardous functions by summing all qualified alignment scores as described in the Methods section. This approach allows for more score contribution for higher identity alignments while still allowing for some contribution for lower identity alignments. We then normalized the scores within each functional category by dividing each value by the maximum value in that functional category. This normalization enables critical hazardous functions that may only be encoded with one or a few CDSs (e.g., a critical toxin) that are absent in nonpathogens to be emphasized within a category and controls for abundance bias within our hazard database across functional categories. For this analysis, we considered only known functions (i.e., the "unknown" functional category Table 1 was excluded) to remove noise from the analysis stemming from sequences with potentially hazardous but unknown functionalities. Figure 3 shows the fingerprints for each of the eight organism groups in the form of heat plots to study visual differences among the various hazard categories. We further analyzed the hazard fingerprint data from

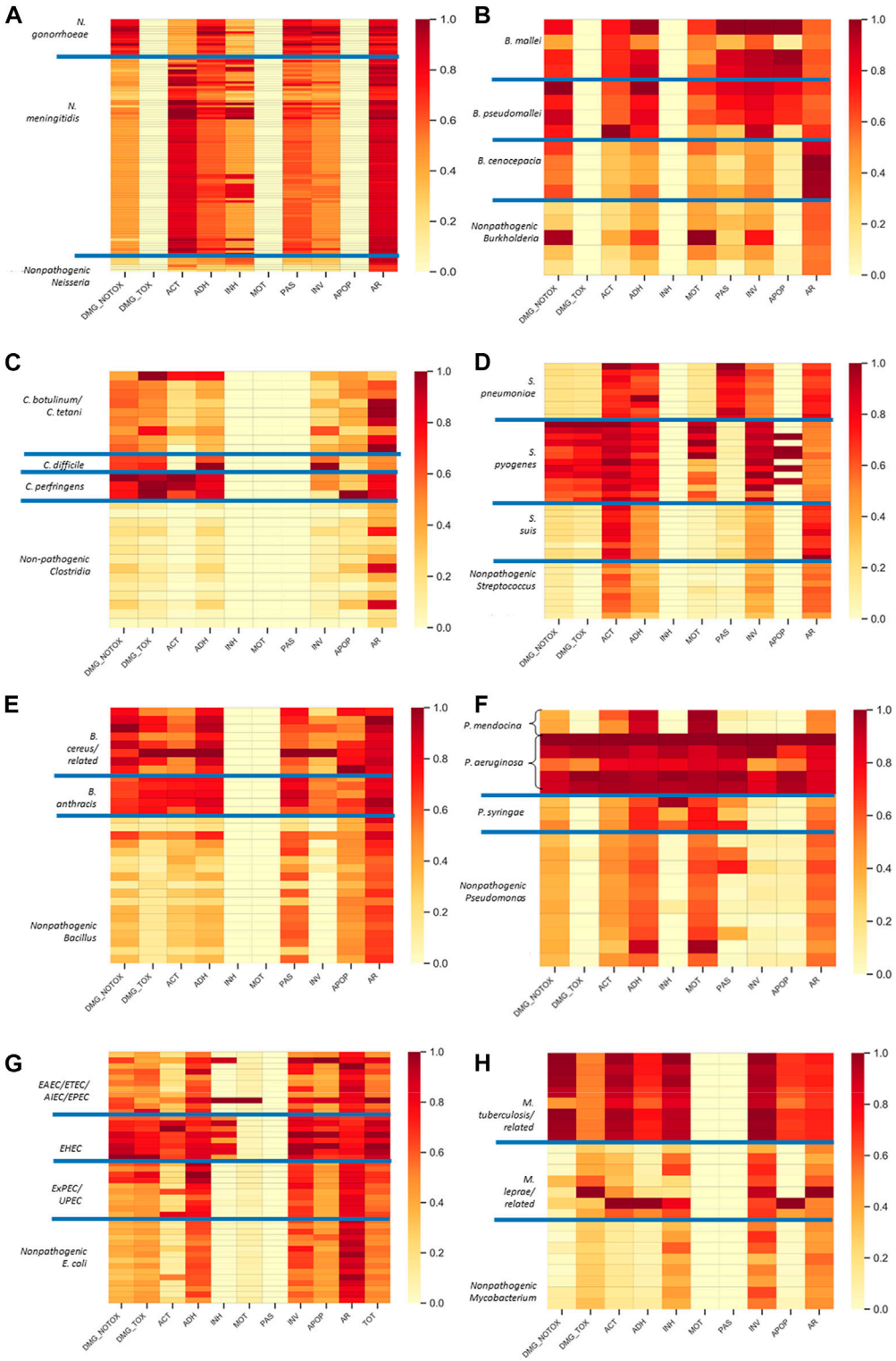


FIGURE 3
Pathogenic species are enriched in hazardous functional categories. Shown are the hazard fingerprints for Neisseria **(A)**, Burkholderia **(B)**, Clostridium **(C)**, Streptococcus **(D)**, Bacillus **(E)**, Pseudomonas **(F)**, *E. coli* **(G)**, and Mycobacterium **(H)**. The fingerprints are shown as rows in a heat plot with the values in each column representing the normalized fraction of CDSs within each functional category (as defined in Table 1). Only the relevant categories from Table 1 are included (i.e., those that provided alignments). The pathogenic subgroups within each organism group are defined in Table 3 and separated by the blue lines on the heat plot. Abbreviations: DMG_NOTOX, damage without toxin activity GO term, DMG_TOX, damage with toxin activity GO term; ACT, active host subversion; ADH, adherence; INH, inhibits host cell death; MOT, motility; PAS, passive host subversion; INV, invasion; APOP, host cell apoptosis; AR, antibiotic resistance; TOT, TOTAL (sum of all other categories).

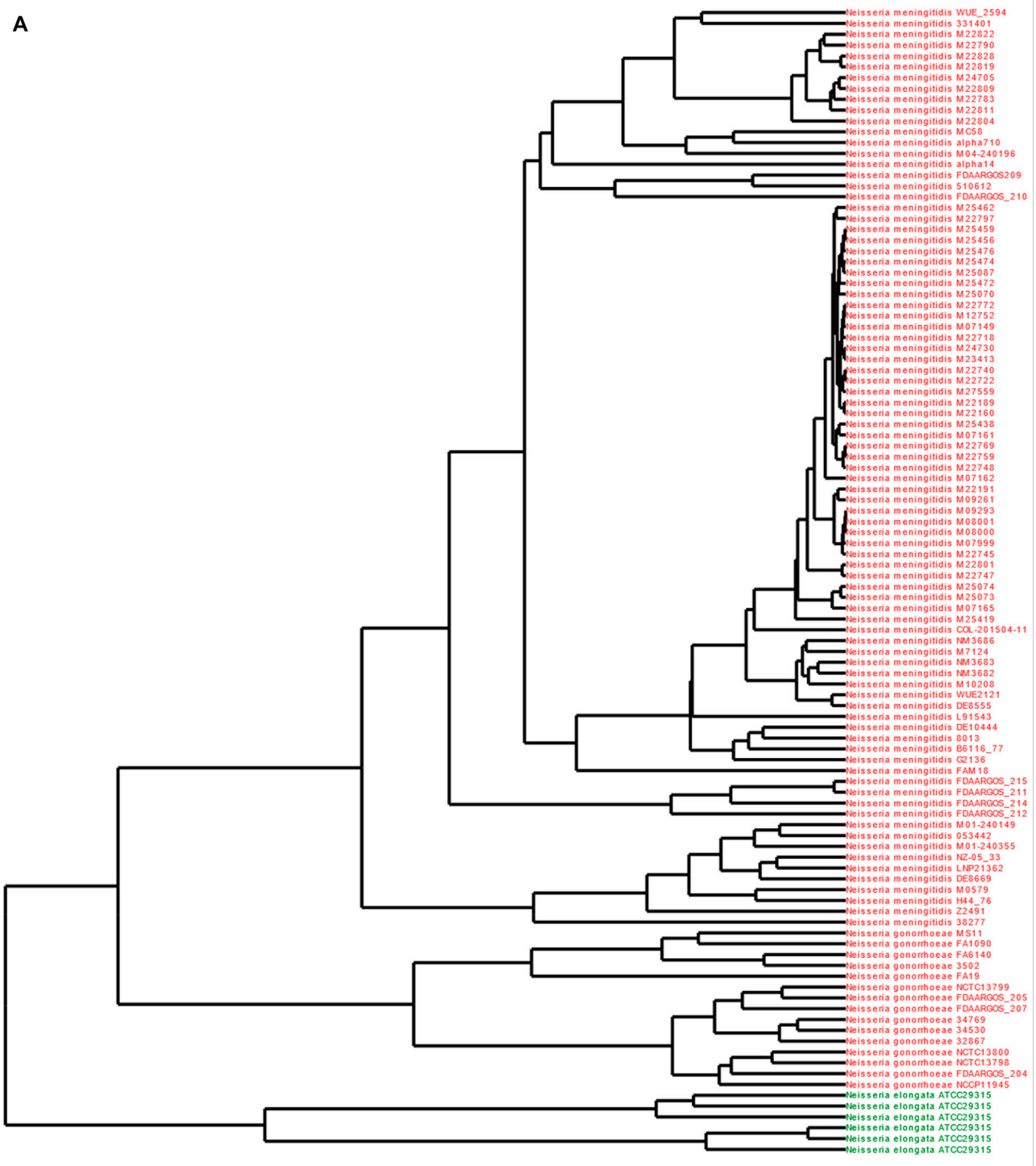


FIGURE 4
Hazardous functions separate pathogens from non-pathogens. Shown are the dendrograms for Neisseria **(A)**, Burkholderia **(B)**, Clostridium **(C)**, Streptococcus **(D)**, Bacillus **(E)**, Pseudomonas **(F)**, *E. coli* **(G)**, and Mycobacterium **(H)**, with pathogenic species colored in red and non-pathogens colored in green. An additional plot for *E. coli*, stratified by the groups shown in Figure 3 is shown in Supplementary Figure S1.

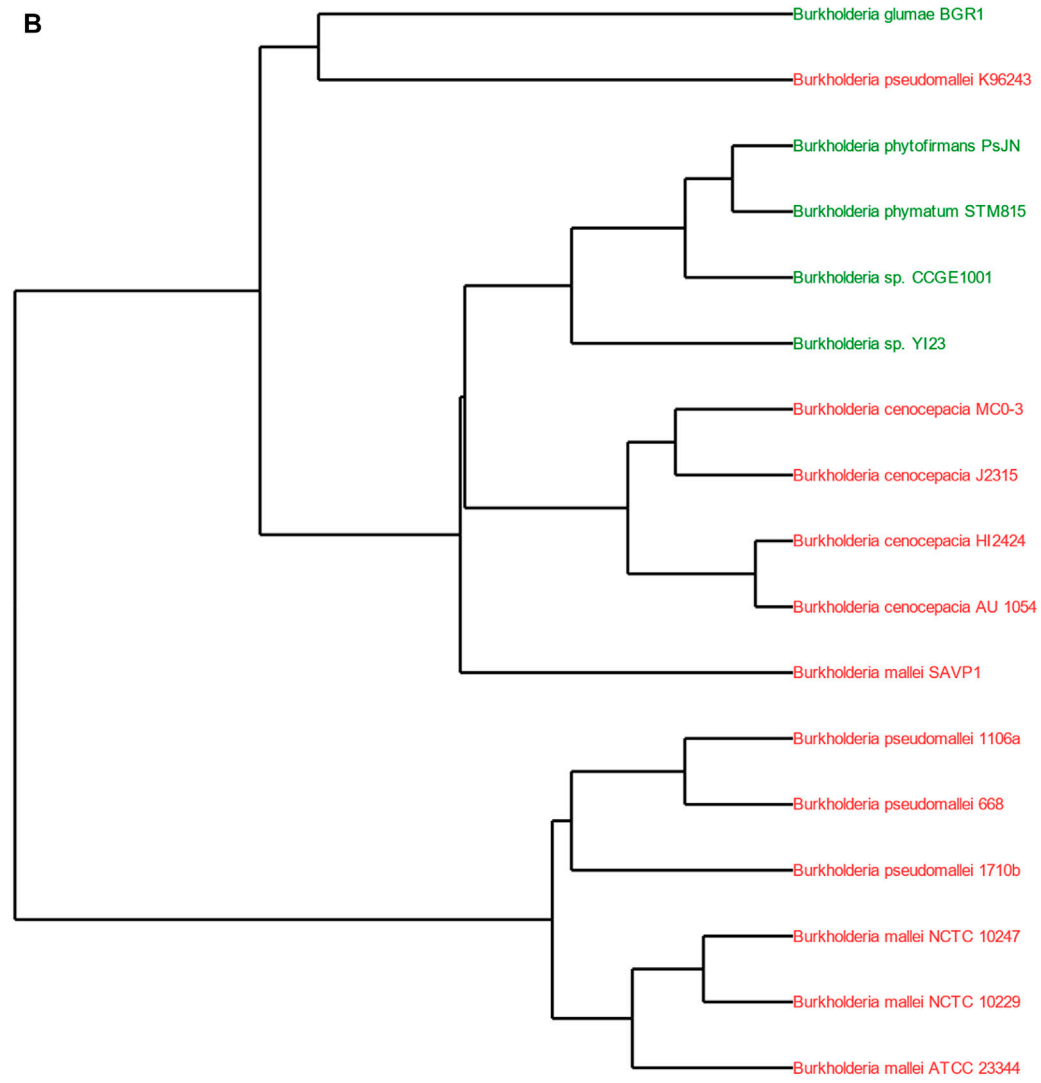


FIGURE 4
(Continued)

the heat plots using agglomerative hierarchical cluster analysis. These clusters were then visualized by plotting dendrograms, where known pathogenic groups were labeled in red, and non-pathogenic in green. For most organisms, hierarchical clustering based on the fingerprint data effectively distinguished between pathogenic and non-pathogenic strains Figure 4).

Overall, the plots demonstrate high levels of hazardous functions in pathogens relative to nonpathogens (Figure 3) and good separation between pathogen and non pathogens (Figure 4). More specifically for the fingerprints, there is good separation across most categories with the exception of antibiotic resistance, and the types of hazardous functions are consistent with literature reports as described below. For example, as shown in Figure 3A, both pathogenic Neisseria groups are enriched relative to the nonpathogen group in adherence, passive host subversion, and invasion functions. Further, the dendrogram demonstrates clear separation between pathogens and nonpathogens (Figure 4A). These findings are consistent with Lu et al., who demonstrated several genes unique to pathogenic Neisseria species that are involved in host immune evasion and adherence (Lu et al., 2019). *N. gonorrhoeae* further contains strains enriched in critical non-toxin damage functions, and *N. meningitidis* is enriched in active host subversion functions such Factor H binding protein (Supplementary Table S1).

Similarly, pathogenic Clostridium groups are clearly separated (Figure 4C), and pathogens are enriched in damage, adherence, and invasion functions relative to the nonpathogen group, with some strains being enriched in active host subversion and apoptosis (particularly the *C.*

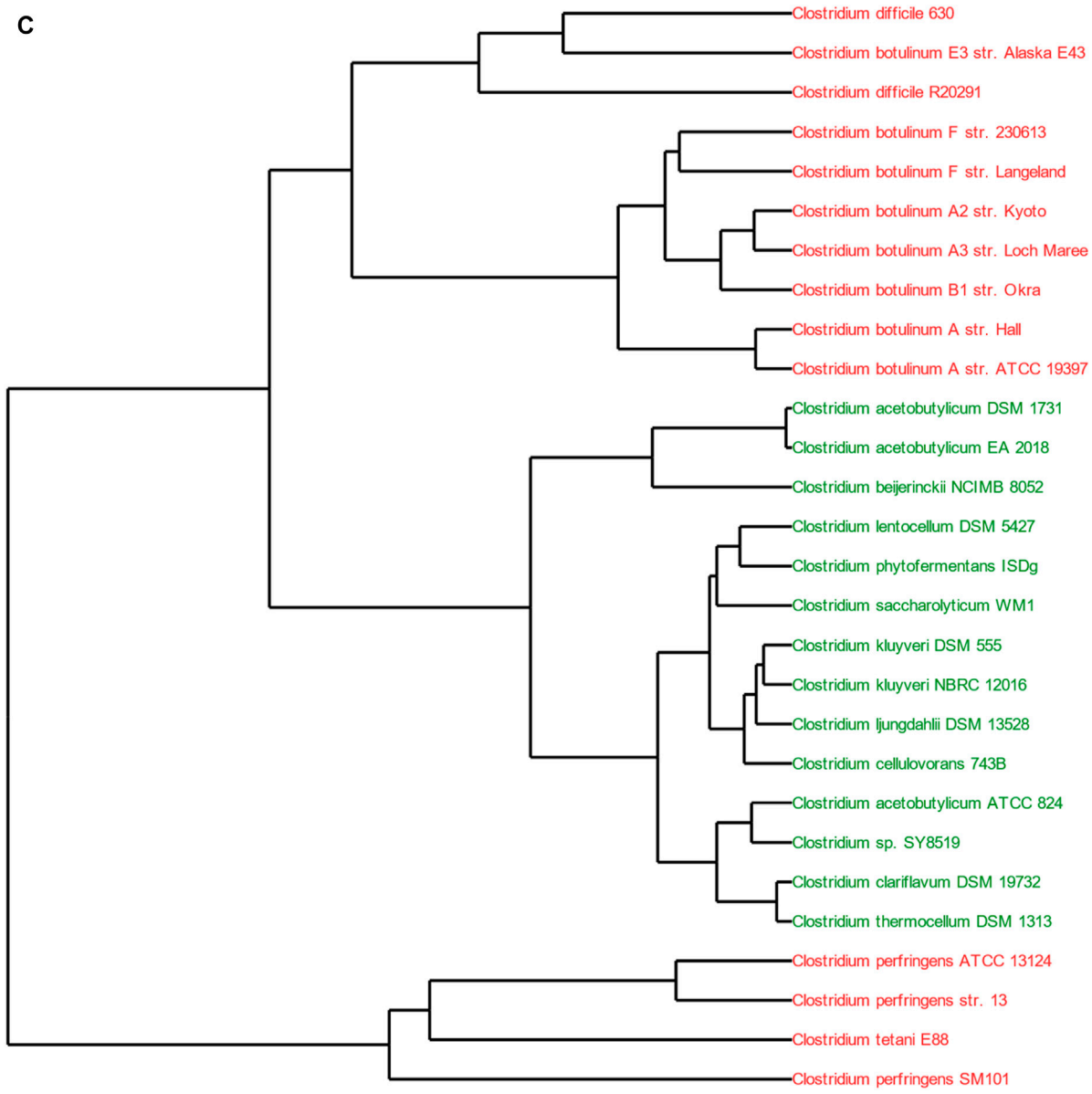


FIGURE 4
(Continued)

perfringens group) (Figure 3C). The most striking of these enriched categories for Clostridium are the damage categories, which is consistent with various Clostridium species producing damage-inducing factors such as toxins as their main hazardous functions, of which some can aggravate the immune response (Supplementary Table S1). For example, *C. botulinum* produces neurotoxins, *C. difficile* produces toxin A, toxin B, and binary toxin, and *C. perfringens* produces over 16 toxins (Awad et al., 2014; Rasool et al., 2017). Because the numbers of toxins produced by *C. perfringens* relative to the other two pathogenic groups is relatively higher compared to the other pathogenic groups, greater delineation between this pathogen group and the nonpathogenic Clostridium group is apparent due to the normalization process.

The Bacillus fingerprints (Figure 3E) demonstrates that Bacillus pathogens are enriched in functions related to damage, active host subversion and adherence relative to their nonpathogenic groups. The fingerprint plot also demonstrates that nonpathogenic Bacillus have antibiotic resistance functions,

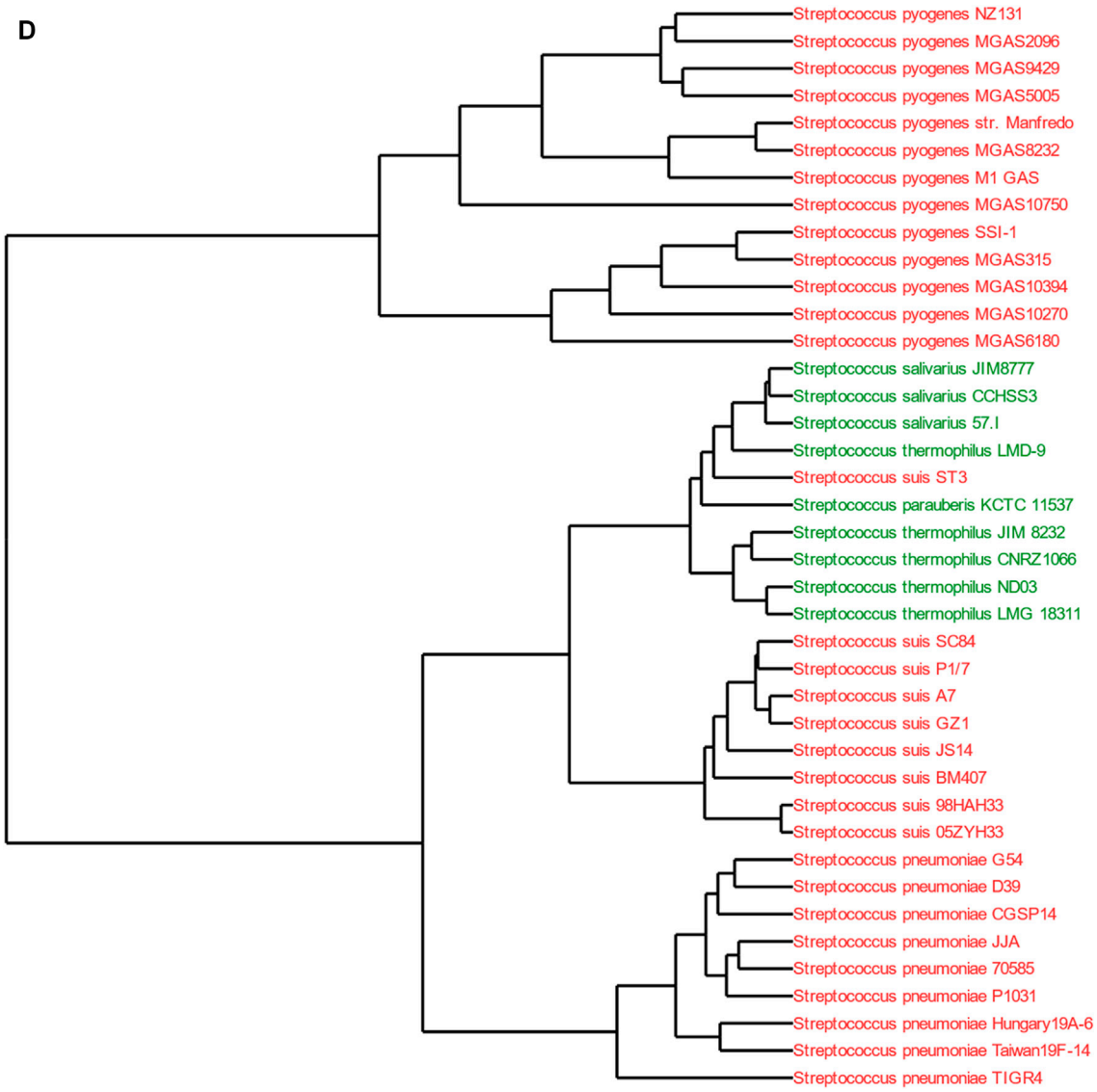


FIGURE 4
(Continued)

which supports other reports (Adimpong et al., 2012; Noor Uddin et al., 2015). For *B. anthracis*, the damage and active host subversion are most clearly delineated from the nonpathogen group, which is consistent with anthrax toxin—composed of protective antigen, edema factor and lethal factor (Supplementary Table S1)—being the major contributor to disease through destruction of host immune cells (Friebe et al., 2016; Visiello et al., 2016). Similarly, *B. cereus* contains factors that promote cell (including immune cell) damage, such as enterotoxins, hemolysins, emetic toxins, and phospholipases (Supplementary Table S1) (Visiello et al., 2016). Taken together, these functions allow separation of pathogens and non-pathogens (Figure 4E), with exception of one presumably non-pathogenic B. cereus strain Q1, an extremophilic strain known for microbial enhanced oil recovery due to production of biosurfactants (Xiong et al., 2009).

The plots also show good separation of some of the Streptococcus species from the nonpathogenic groups, particularly *S. pyogenes* (Figures 3, 4D). *S. pyogenes*—known as Group A Streptococcus clinically—has several factors enabling invasion, adherence, and motility within host cells, but perhaps the most important factors contributing to pathogenicity of *S. pyogenes* are the few proteins leading to direct damage (e.g., streptolysins O and S, and exotoxins A and C) and host evasion (e.g., IgG-degrading enzyme and Protein M) (Hamada et al., 2015). These critical functions are apparent

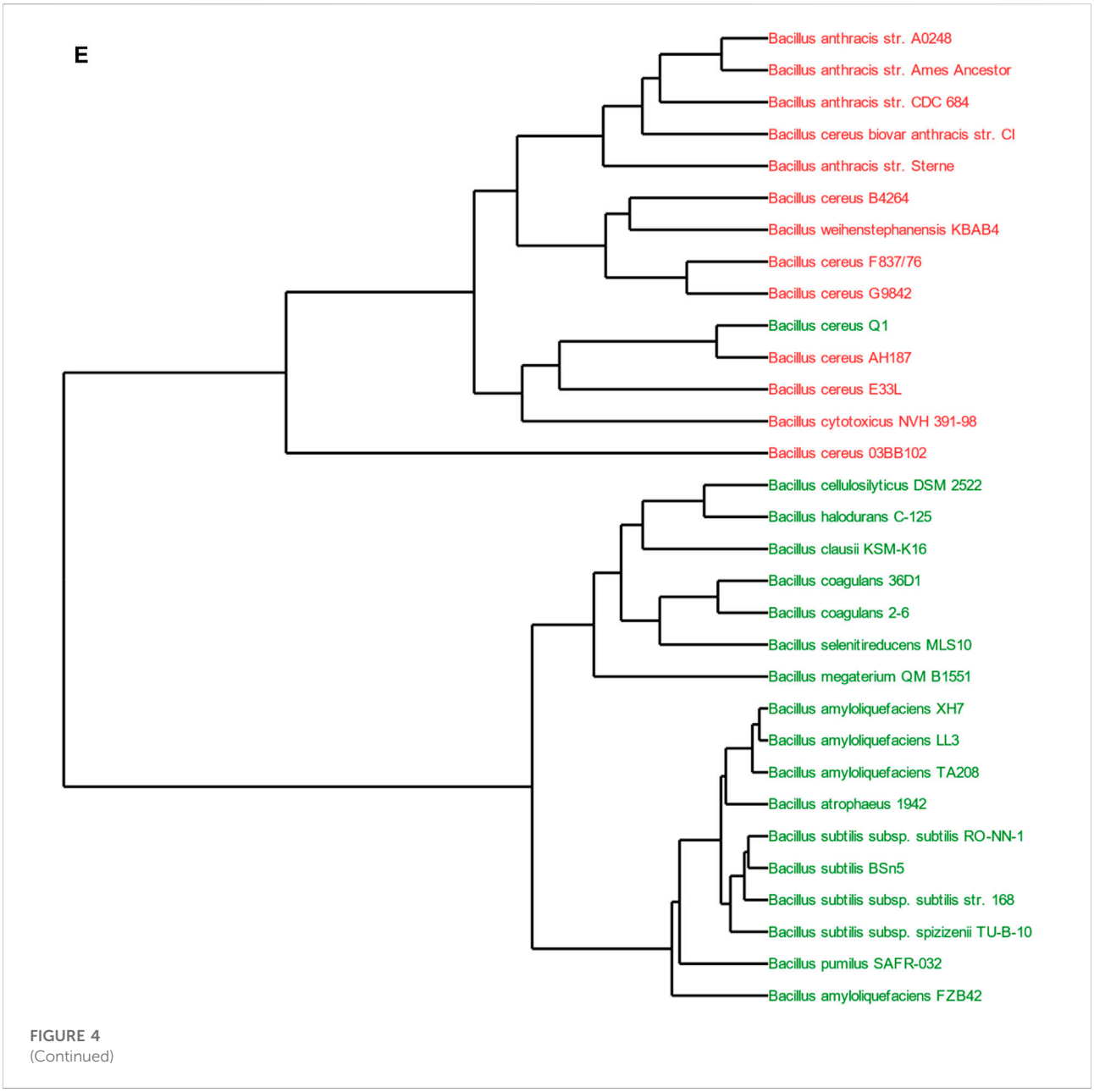


FIGURE 4
(Continued)

in the heat plot as well as Supplementary Table S1. Less defined separation is apparent between the nonpathogenic group and the *S. pneumoniae* or *S. suis* group with a few exceptions. For example, antibiotic resistance factors show some delineation from the nonpathogen and *S. pneumoniae* or *S. suis* groups, which is consistent with the emergence of antibiotic resistance strains in these species (Nuermberger and Bishai, 2004; Yongkiettrakul et al., 2019). Further, enzymes leading to *S. pneumoniae* cell wall decoration that enable immune system avoidance (Mitchell and Mitchell, 2010) likely contributes to this group being separated from the other groups within the passive immune subversion category. *S. pneumoniae* and *S. suis* also express critical damage factors, such as the PLY pore-forming toxin (Mitchell and Mitchell, 2010) and hemolysins (Haas and Grenier, 2018), respectively, which—while not very apparent in Figure 3 due to high levels of the damage functional category in *S. pyogenes*—are identified as critical factors in Supplementary Table S1. Taken together, these hazardous functions enable good separation of pathogens from non pathogens. One exception is the pathogenic strain *S. suis ST3*. According to this Hu et al., this strain is missing a large pathogenicity island (Hu et al., 2011), which is the likely cause of lack of separation.

Like Streptococcus pathogens, Mycobacterium pathogens, particularly tuberculosis-causing Mycobacteria, are separated

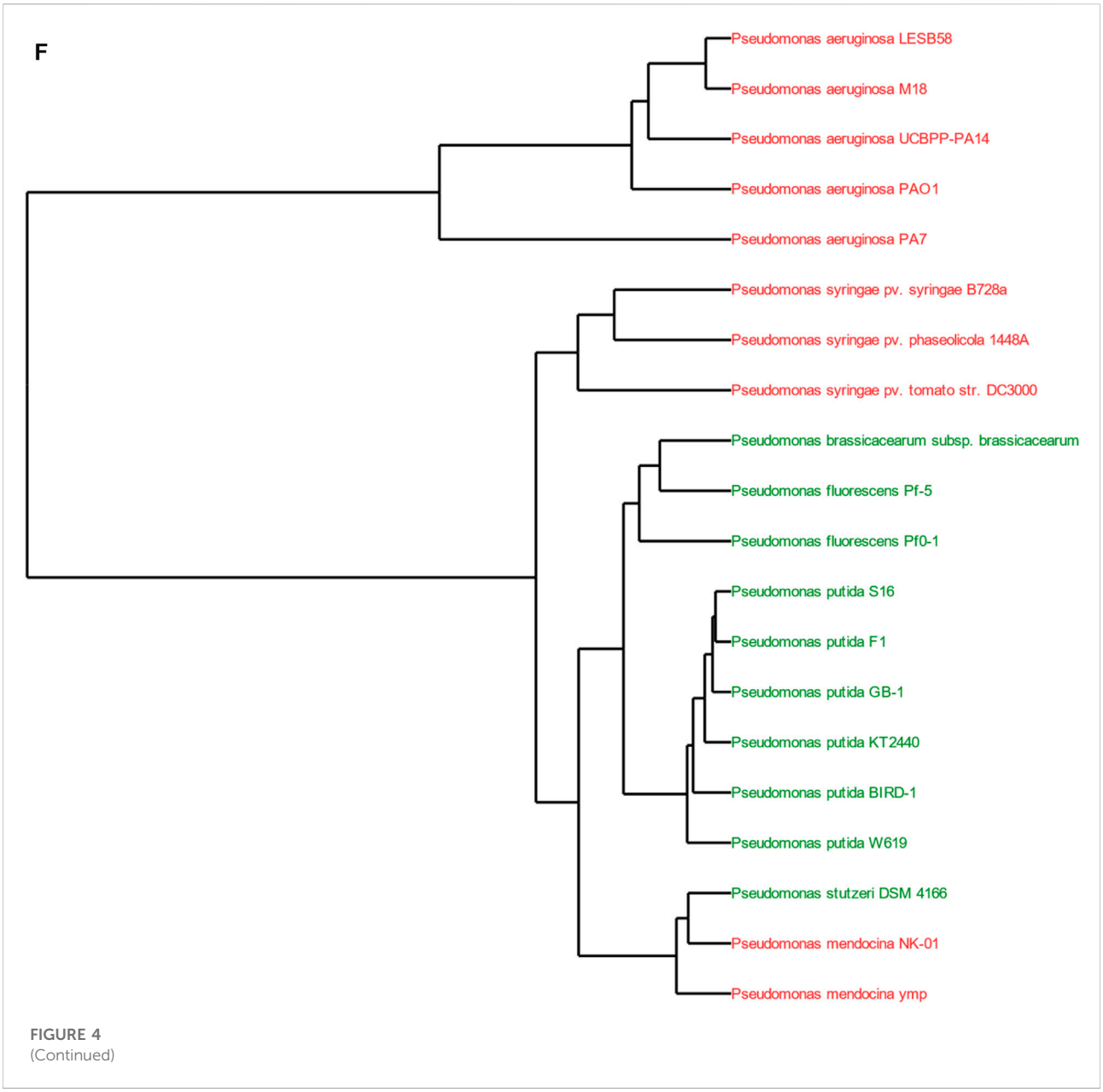


FIGURE 4
(Continued)

well within specific hazardous categories (Figure 3H) and separate well from non-pathogens (Figure 4H). One exception is *M. abscessus ATCC 19977*, a pathogen that clusters with non pathogens. This finding is actually consistent with another report, which demonstrated that this strain clusters with other non-pathogens based on whole proteome analysis (Zakham et al., 2012). In general, we found that *M. tuberculosis* strains are enriched in active host subversion, adherence, and apoptosis categories relative to the nonpathogen group, which is consistent with the fact that *M. tuberculosis* virulence largely depends on the organism's ability to infect host cells and evade the host immune response (Forrellad et al., 2013). The plot additionally shows that damage factors contribute to differences compared to the nonpathogen group, which supports the fact that *M. tuberculosis* requires damage factors such as adenylate cyclase (Supplementary Table S1) for virulence (Agarwal et al., 2009). In contrast to *M. tuberculosis*, less separation is apparent for the *M. leprae* and related group. This observation is likely because only 24 of the 339 Mycobacterium hazardous functions contained in our database are from the *M. leprae* and related group, and the CDSs from this group may not have enough homology to hazardous functions from *M. tuberculosis* strains to be relevant in our analysis.

Similar to the Mycobacterium analyses, some hazardous categories are emphasized for *E. coli*, although our analysis

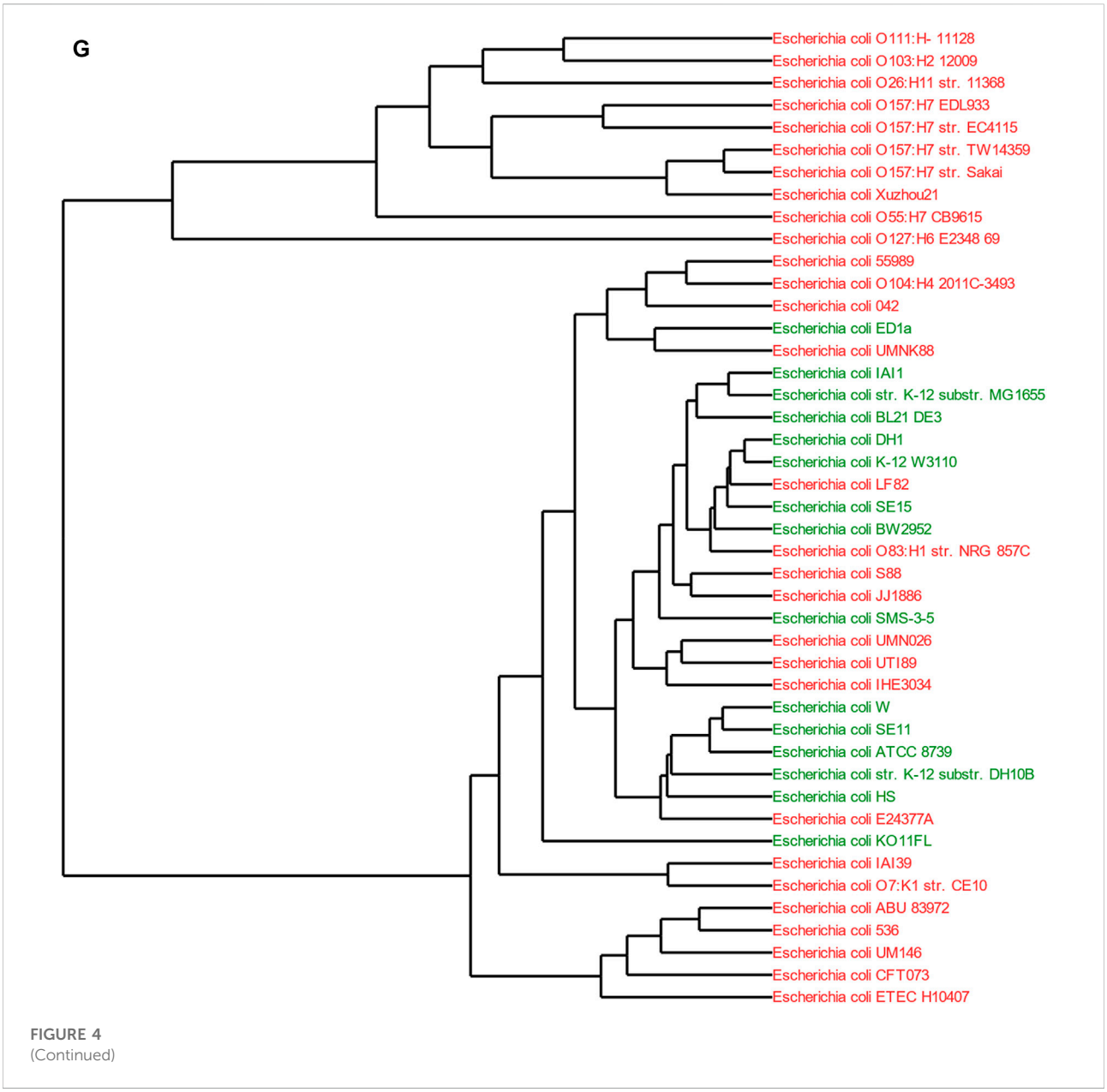


FIGURE 4
(Continued)

was not able to clearly separate all pathogenic groups (Note: Figure 4G colors and labels the dendrogram based on pathogenic and non-pathogenic strains, whereas Supplementary Figure S1 colors by pathogenic and non-pathogenic group). Since infections caused from intestinal pathogenic *E. coli* (IPEC) are distinct from infections caused extraintestinal pathogenic *E. coli* (ExPEC, including uropathogenic *E. coli*) (Kohler and Dobrindt, 2011), we separated with *E. coli* pathogenic strains into IPEC strains—including a group of enterohaemorrhagic *E. coli* (EHEC) and non-EHEC strains (EAEC/ETEC/AIEC/EPEC)—and ExPEC strains. While EHEC strains are clearly separated (Supplementary Figure S1), ExPEC strains could not be separated as well, likely because these strains can belong to the normal (nonpathogenic) gut flora and share large portions of their genome with nonpathogenic strains (Kohler and Dobrindt, 2011). In contrast to the ExPEC strains, the IPEC strains—particularly the EHEC strains—show greater relative abundance of damage functions (Figure 3E). This observation supports that fact that functions that contribute to host cell damage are critical to IPEC pathogenesis, such as enterotoxins and shigatoxins (within ETEC and EHEC strains, respectively) as well as functions leading to attaching and effacing lesions (Welch et al., 2002; Kaur et al., 2010; Nguyen and Sperandio, 2012). The EHEC group is also further differentiated from the other IPEC strains within the active host subversion and inhibits host cell death categories, which is a hallmark of EHEC strains (Ho et al.,

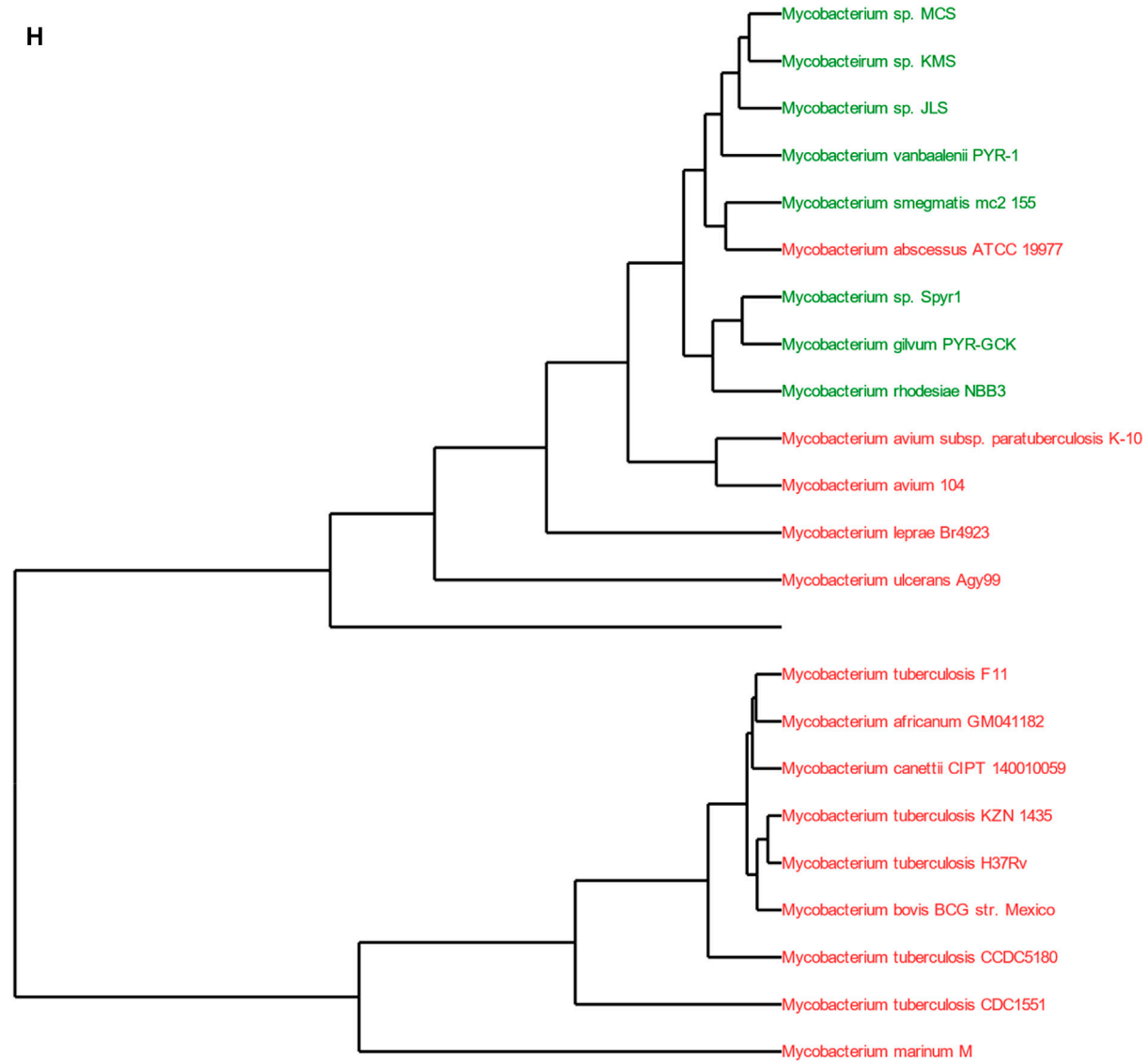


FIGURE 4
(Continued)

2013). IPEC strains also elicit aggressive adherence functions to enable pathogenicity (Kaur et al., 2010), but our methods did not enable clear emphasis of this category in pathogenic strains compared to nonpathogenic stains, likely due to the ubiquitous nature of adherence functions.

For Burkholderia, our analysis enables good separation, with the exception of B. pseudomallei K96243, a pathogen that clusters with non-pathogens (Figure 4B). Previous analysis of the genome of this strain noted high similarity to Ralstonia solanacearum, a plant pathogen (Holden et al., 2004), which is consistent with this strain clustering with B. glumae and B. phytofirmans (plant colonizers) in our analysis. *B. mallei* and *B. pseudomallei* are intracellular pathogens that use numerous virulence factors that enable host cell survival, such as invasion and immune evasion factors (Galyov et al., 2010; Memisevic et al., 2014), which is apparent in Figure 3B. These organisms also contain key factors such as BimA, hemagglutinin, PilA, which are involved in invasion, damage, and adherence, respectively (Sarovich et al., 2014) that enable emphasis of these categories in the plot. In contrast to *B. mallei* and *B. pseudomallei*, the only enriched functions for *B. cenocepacia* are antibiotic resistance and non-toxin damage functions, but this may be an indication of lack of coverage in our database (only 2 of the 141 hazardous Burkholderia functions are from *B. cenocepacia*). However, this finding is consistent with the fact that *B. cenocepacia* clinical strains isolated from cystic fibrosis patients

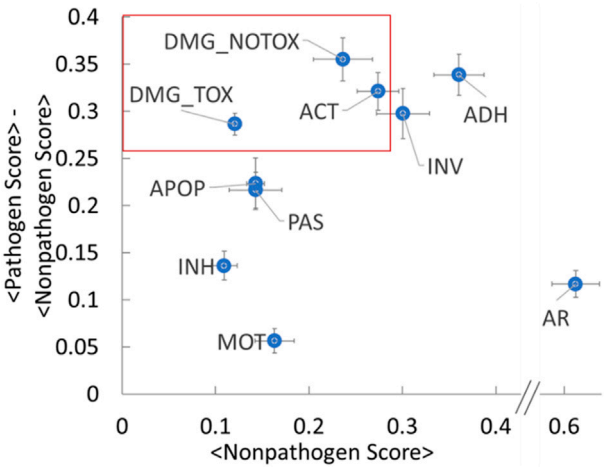


FIGURE 5
Pathogen and Nonpathogen Fingerprint Scores Reveal Stratification Among Functional Hazard Categories. The plot shows a difference in average scores between the pathogens and nonpathogens (*y*-axis) as a function of the average nonpathogen score (*x*-axis) for the functional categories from the heat plots in Figure 3. The error bars represent the standard error. The abbreviations are the same as Figure 3.

can be resistant to antibiotics and contain several lipases and proteases to illicit tissue damage (Mahenthiralingam and Vandamme, 2005). Noticeably, *B. glumae* (third row from the bottom in Figure 3B demonstrates some pathogenic signatures, which is consistent with research demonstrating that this species can be a rice pathogen (Pedraza et al., 2018). This species was originally considered a nonpathogen based on the dataset published by Cosentino et al. (Cosentino et al., 2013), suggesting that our methods may enable identification of misannotated organisms.

Finally, some separation is also apparent for Pseudomonas species, but the patterns are not as consistent across strains as the other pathogens (Figures 3, 4F). Pseudomonas species pathogenic to humans (*P. aeruginosa* and *P. mendocina*) have a wide variety of virulence factors (Goldberg, 2010), but the patterns are different between the two species, and these two groups are completely separated in the dendrograms (Figure 4). For example, both *P. aeruginosa* and *P. medocina* have several proteins contributing to adherence and motility (Supplementary Table S1), but these types of functions can occur in nonpathogenic species as well. In contrast, invasion factors, host cell subversion factors, host cell apoptosis, and damage factors are relatively unique to *P. aeruginosa* strains (Figure 3 and Supplementary Table S1), which is consistent with experimental evidence (Shaver and Hauser, 2004; Dulon et al., 2005; Casilag et al., 2016; Basso et al., 2017; Reboud et al., 2017). Antibiotic-resistance functions are higher in *P. aeruginosa* pathogenic strains as well, which is consistent with the clinical prevalence of antibiotic resistant strains (Jacoby and Munoz-Price, 2005). For plant pathogens, our methods result in some separation of *P. syringae*—a plant pathogen—from nonpathogenic Pseudomonas species overall (Figure 4), and within the inhibits host cell death functional category (Figure 3). These observations may be driven by the fact that only 2 of the 175 Pseudomonas hazardous functions contained in our database are from *P. syringe*.

Toward application of the methodology and resulting functional hazard database

The fingerprint analysis presented in the previous section demonstrates that categorizing hazardous functions allows the importance of the gross functionalities (i.e., the functional metadata categories in Table 1) to differentiate nonpathogenic groups from pathogenic groups for both gram-negative and gram-positive bacteria. As further demonstration of our methodology and database with an eye toward the utility of our method for biosafety assessments, we sought to determine the relative hazard level of each functional category. Logic suggests that two parameters play a large role in such a relative ranking: 1) the magnitude of the category's increase in relative abundance compared to nonpathogens and 2) the relative abundance of the category in nonpathogens. As a simple measure of these parameters, we leverage the data used to generate the heat plots to calculate an average score for each of the functional categories for the nonpathogen and pathogen groups. Figure 5 shows a plot of the difference in average scores between the pathogens and nonpathogens as a function of the average nonpathogen score. The points on the upper left quadrant of this graph thus represent highly hazardous categories that 1) have a relatively large difference between the pathogen and nonpathogen scores and 2) have a low background signature (i.e., low nonpathogen score). For example, these results suggest that the damage (with and without toxin activity) and active host subversion categories have relatively high pathogen-nonpathogen difference scores (e.g., >0.25) with low nonpathogen scores (e.g., <0.3) (red box in Figure 5). Such an analysis demonstrates a potential ranking system for "sequences of concern," and may enable a foundation for a risk-based approach for biohazard assessments for designed organisms. As mentioned above, more hazardous functions that do direct damage to a cell or those involved in avoiding the host immune system rank more highly than less hazardous functions such as adherence and motility. Thus, the damage and active host subversion categories may present a higher hazard relative to other categories for biohazard analysis, for example. Generalizing this approach across all functional categories and all organism types may provide an objective foundation for biohazard analysis of novel organisms.

Discussion

While the methodology and database presented here has two immediate uses—1) biosecurity screening assessments of synthetic

genes and 2) partial biosafety assessments for bacterial genomes—future work should build upon this foundation to provide comprehensive biosecurity and biosafety assessments for the synthetic biology community. We envision a future in which any novel biodesign can be assessed through a function-based paradigm that requires only genomic sequences. This paradigm is in contrast to current biosafety assessments that rely on phenotypic information from well characterized organisms to classify organisms into Biosafety Levels, for example, which provides researchers with an understanding of the level of pathogenicity, transmissibility, and other characteristics of the organism (U.S. Department of Health and Human Services, 2014). However, as the genomes of new biodesigns begin to deviate further and further from these well characterized organisms, biosafety levels become less and less clear, thus necessitating in silico genome characterization methods. Where traditional biosafety assessments are limited to known pathogens with no or minimal bioengineered parts, with future development, our framework may enable assessment of seemingly limitless potential for biodesigned organisms. In this discussion, we elaborate on the issues with the current paradigm, how our approach begins to shift the paradigm, and the future work needed to provide a complete paradigm shift.

Progress in bioengineering, synthetic biology, and computational science is enabling artificial creation (*de novo* genetic synthesis) of whole organisms, including viruses (Blight et al., 2000; Cello et al., 2002; Smith et al., 2003; Oldfield et al., 2017; Noyce et al., 2018) and bacteria (Gibson et al., 2010; Hutchison et al., 2016), as well as recombinant production, viral reverse genetics, rational design, design from standardized DNA components (e.g., Biobricks), and/or modular protein assembly (e.g., SpyTag or SpyCatcher (Khairil Anuar et al., 2019)). Such technologies have led to exponential growth of publications based on synthetic biology since 2000, and larger throughput per synthetic biology lab (Raimbault et al., 2016). Further yet, DNA synthesis is becoming more distributed, for instance, with the availability of DNA printers such as the BioXp system from Codex DNA. As breakthroughs are made to realize the promise of synthetic biology, the creation of novel sequences may expand even more, and such growth is difficult to monitor. Although the numbers of new natural strains being discovered is accelerating fairly linearly (Suzek et al., 2015; RefSeq, 2019), the production of bioengineered strains may be growing exponentially, as many of these sequences are not publicly available. This rapid progress in bioengineering has created a gap in current biosafety practices that requires a framework to understand the potential hazards posed by functional building blocks. We have provided empirical data that demonstrates a function-centric paradigm for identifying and classifying hazardous biological parts. The functional classification of sequences is based on coarse hazardous functions encoded by organisms, such as functions contributing to pathogenicity, toxin and drug production, and immune regulation.

The methodology demonstrated here can immediately be used for partial biosafety assessments for bacterial genomes for classification of pathogens and non-pathogens using functional hazard fingerprints. Future iterations of the method should involve testing both previously characterized organisms and novel organisms (i.e., those not contained in the database and/or novel biodesigns with known phenotypes) in order to characterize a variety of biosafety-related characteristics (not just pathogenic/nonpathogenic) from various domains of life beyond bacteria (e.g., viruses and fungi). As we demonstrated in Figure 4, hierarchical clustering achieves a high level of separation between pathogen and nonpathogen organism group members using a simple alignment with default parameters against our curated database. This approach is in contrast with more complicated, manual annotation and phylogenetic analysis that require time-consuming, expert interpretation. Even outlier pathogens that cluster with nonpathogens like *S. suis ST3* have characteristics that explain why they do not cluster with other pathogens; for example, as noted *S. suis ST3* clusters with nonpathogenic organisms but is missing a pathogenicity island, which likely contains several hazardous functions. Similarly, outlier nonpathogens that cluster with pathogens such as *B. cereus Q1* can be explained as well. The genome for this organism contains genes encoding for enterotoxins (NCBI accessions ACM12308, ACM12309, and ACM12310) involved in damage and adherence, lipid transferases involved in passive and active immune subversion (accessions ACM11963 and ACM12924) and antibiotic resistance (accessions ACM12845 ACM12455). Thus, if used for assessments of pathogenicity, false negatives (due to lack of hazardous functions and/or presence of previously uncharacterized functions) and false positives (due to the presence of hazardous pseudogenes and/or non-hazardous sequences with high homology to hazardous functions) could occur depending on the thresholds used for classification. However, the success of this approach demonstrates the native utility of the hazardous function database and that further refinements in fingerprinting approach are both attainable and could be an effective diagnostic approach to classifying unknown organisms.

As documented in Table 3, some pathogens have higher coverage in our database than others, and thus comparison across pathogenic groups should be interepreted appropriately. Differences between a pathogen and nonpathogen in one organism being less pronounced relative to another organism group could be due to large functional differences, but it could also be due to lack of database coverage. For example, the fact that *M. tuberculosis* pathogens have higher numbers of hazardous functions compared to *N. gonorrhoeae* does not mean necessarily that *N. gonorrhoeae* is relatively more pathogenic compared to its nonpathogenic conterparts than *M. tuberculosis*; this result may be driven by the larger coverage of Mycobacterium sequences within our database. Determination if our approach can be used

to elucidate levels of pathogenicity based on a collection of hazardous functions warrants further exploration. Such an application may have utility beyond biosafety assessments, such as emerging and recurrent disease identification. As recently stated by others, new approaches are needed to address emerging diseases (Reperant and Osterhaus, 2017), particularly as surveillance and diagnostics improve across the globe. We propose that a function-based paradigm provides a foundation to meet this need, and such approaches have already shown success. In this study, we leveraged data from Cosentino et al., who developed methods to classify bacterial pathogens from nonpathogenic bacteria based on protein families (Cosentino et al., 2013), which have a direct link to function (Pearson, 2013). Beyond bacteria, others have shown that sequence differences leading to functional differences are critical determinants of pathogenicity for viruses and fungi such as influenza virus (Ebrahimi et al., 2014; Straus and Whittaker, 2017), African Swine Fever Virus (Chapman et al., 2008), Zika virus (Shah et al., 2018), Colletotrichum spp. (Vieira et al., 2019), and Geosmithia spp. (Schuelke et al., 2017). Thus, development and generalization of models may aid in the shift from organism to function-based classifications for all types of infectious disease. For example, a logical extension of the study presented here would be to determine if similar results can be obtained if we leveraged our entire database (not just specific subsets of hazardous functions from selected bacteria), such that a prior knowledge of the organism in question is not needed.

In addition to the immediate use of our methods for predicting pathogenicity of bacteria, the method and database also has immediate use for screening individual gene sequences. The example application of our methodology and database to stratify sequences are promising, but the results suggest more granular functional categories may be needed to enable use for more pointed biosafety assessments. Granular metadata for protein sequences are available from several databases that are cross-referenced within the UniProt Knowledge Database (UniProt, 2019b), such as Gene Ontology (GO) terms (Ashburner et al., 2000; The, 2019), Interpro terms (Mitchell et al., 2019) and sequence features (e.g., motifs, regions, mutation impact, etc.). GO terms provide a graphical representation of molecular functions, biological processes, and cellular components of gene products and their relations among each other (Ashburner et al., 2000; The, 2019). We leveraged the "toxin activity" GO term within our framework, but further use of GO terms may enable better stratification of hazardous sequences.

Our results may also improve if host information is considered. Recent efforts, such as ViralZone (ViralZone, 2019) and the proposed PathGO (IARPA. Broad Agency Announcement, 2016) are providing better GO terms for host-pathogen interactions that may prove valuable for function-based hazard classification. Casadevall proposed a damage response framework (Casadevall and Pirofski, 2003) that is founded on the simple principle that microbial pathogenesis is "the outcome of an interaction between a host and a microorganism" measured by damage to the host. Current knowledge suggests pathogens interact with the host in a variety of ways, including mimicking host activities, leading to a lack of host cellular control (Knodler et al., 2001; Stebbins and Galan, 2001; Smatti et al., 2019), but documentation of these data in a machine-readable format is sparse. Two potentially useful sources of information that are cross-reference in UniProt are IntACT (Hermjakob et al., 2004), which provides protein-protein interaction data, and Reactome (Reactome, 2019), which provides functional metadata associated with biological pathways. An initial analysis of our hazardous functions suggests that <2% of protein accessions in our database have at least one interactor in IntACT database, and 58% of the interacting proteins are human proteins. These human proteins represent 3% of the total reactome metadata. In addition to IntACT, specific (host-pathogen) protein-protein interaction information is available from Biogrid (Oughtred et al., 2019), String (von Mering et al., 2005) and other databases, but information is sparse. However, as high-throughput experimentation becomes more commonplace, information contained in these databases can be leveraged for hazard analyses. Specifically, further expansion of these databases for hazardous sequences may be needed for impactful analysis and utility into a function-based biosafety assessment.

In addition to hazards that may impact hosts such as humans, livestock, and crops, other living hosts and non-living "hosts" of economic importance should be considered as well for other pointed biosafety assessments. For example, when considering safety assessments for novel bio-based fertilizers and/or biopesticides, hazards with economic impact potential beyond those that effect crops and livestock may need to be considered. For example, of the world's ~250,000 flower and seed-producing plant species, between 78% and 94% require pollinators for fertilization ("FAOSTAT" Food and Agricultural Organization www.fao.org), with bees accounting for pollination of approximately 30% of the world's food supply (Klein et al., 2007). Bee colonies can collapse from fungal, bacterial and viral outbreaks, such as those caused by the picornavirus-like deformed wing virus (DWV) and the ectoparasitic mite Varroa destructor (Tehel et al., 2019). Similarly, functions that could negatively impact non-eukaryotic or non-living "hosts" of economic importance should also be considered for tailored safety assessments. For example, under the current paradigm of biosecurity, biodesigns have been created that could potentially impact biomanufacturing supply chains (Abdulamir et al., 2014), control of pharmaceuticals (Galanie et al., 2015; Nakagawa et al., 2016), and crude oil supplies (Xu et al., 2018). Thus, as bioengineering rapidly progresses, safety practices need to keep pace to not only protect humans, livestock, and crops, but also protect infrastructure of critical economic impact.

Expansion of sequences and metadata may thus improve upon our foundation for biosafety practices of the bioengineering-centric future. Our methods and database reported here provide an understanding of the hazard posed

by "parts" of the organism, such that a foundation can be set to understand the hazard of the "whole." For example, *P. aeruginosa* has numerous hazardous functional parts including those contributing to adherence (type 4 pili and flagella for interacting host cells), invasion (T3SS), host cell subversion (biofilm formation, stimulation of proinflammatory response, and disabling of protease activity receptor-2), host cell apoptosis (exotoxin A stimulation of programmed cell death), damage (and cytotoxic effector proteins) and antibiotic resistance (beta-lactamases) (Shaver and Hauser, 2004; Dulon et al., 2005; Jacoby and Munoz-Price, 2005; Casilag et al., 2016; Basso et al., 2017; Reboud et al., 2017; Shen et al., 2017). While many of the hazardous functions of *P. aeruginosa* are known, a biodesign created with similar hazardous functions may not be identified under the current organism-centric paradigm. We must now build upon our methods developed using the engineering-like principle of pathogens being an organized assembly of functional hazards. Using this paradigm, we can then classify groups of sequences that compose a novel pathogen, thus enabling generalized function-based biosafety assessments for novel organism-level biodesigns for all types of applications.

Methods

Hazardous function database

Hazardous functions were identified from publicly available literature and databases (e.g., Supplementary Table S2) as those that have a function that impacts human and non-human hosts of high economic value as described in the Results section. We defined a hazardous function as a set of one or more protein sequences and associated manually curated metadata (Table 1). Each hazardous function can contain one or more functional categories. A hazardous function is only included in the database if its sequence encodes for a verified function based on experimental data from the literature or (in cases such as some select agent viruses where experimental data do not exist) based on homology to a sequence with verified function. Protein sequences were retrieved from UniProt when available or manually entered based on literature documentation. Functional metadata categories were developed based on panel discussions of high-level hazardous functions used by pathogens and organisms producing toxins, drugs, and bioregulators. For hazardous functions in the "damage," category, the toxin activity gene ontology term (GO:0090729) was used to distinguish toxins from non-toxins. Further, for sequences involved in the biosynthesis of small molecule toxins or drugs, hazardous functions were annotated with the step removed from the final product (e.g., last step, second-to-last step) based on pathway information as described in the literature and/or on Metacyc (Caspi et al., 2018).

Identification of hazardous coding sequences from bacteria

To validate the above methods and resultant database, we compared pathogenic and non-pathogenic strains against our functional hazard database. For this exercise, we compiled coding sequences (CDSs) from human and animal pathogenic and nonpathogenic strains based on the references outlined in Table 3. For each identified reference, pathogenic and nonpathogenic strains were reviewed; if a nonpathogenic strain was revealed as a pathogenic strain to a host of interest (or vice versa) based on other literature sources (e.g., a source published after the primary reference), it was removed from the analysis. Further, if an organism has known plasmids with sequences not deposited in NCBI, it was removed from the analysis. Pathogenic species or strains from each organism group were further stratified into subgroups based on species groups or disease-causing metadata (Table 3, column 4) for comparative purposes. CDSs, including those from chromosomal accessions and associated plasmid accessions were downloaded using NCBI's Batch Entrez online tool (NCBI, 2019). Plasmids were included since genetic determinants of bacterial virulence are often carried on mobile elements such as transposons and plasmids (Zaluga et al., 2014). Each strain's CDSs were defined by those contained within all chromosomes and plasmids associated with that strain. For each organism group, CDSs were aligned against a database of hazardous functions from its same genus using the Local Aligner for Massive Biological DatA (Lambda) (Hauswedell et al., 2014) version 2–1.9.5 using default settings. The alignment score, A, was defined as

$$A = Percent\ Identity \times Percent\ Hazardous\ Sequence\ Coverage \quad (1)$$

As discussed, we define the minimal alignment score for a CDS to be a hazardous function as 40% based on the thresholds used to define UniRef50 clusters. We then determined the fraction of hazardous CDSs (total number of CDSs in each strain normalized by the strain's total number of CDSs) and averaged the results of each strain within each pathogen and nonpathogen group.

Hazardous function fingerprinting

To determine a hazard function fingerprint for each strain, the alignment scores, A, for each CDS (to the genus-specific hazardous function database) were summed for each functional category then normalized to the maximal value across all pathogen and nonpathogen groups within that functional category. If a strain did not have a CDS with an alignment to the hazardous function, A was set to zero. Since each hazardous function can contain one or more functional categories, we defined the fingerprints as follows.

For each CDS set of alignment results (i.e., one CDS to one or more hazardous functions), the maximal A for each functional category (Table 1) was tabulated. For example, suppose CDS_i aligns to $Hazardous\ Function\ Sequence_1$ and $Hazardous\ Function\ Sequence_2$ with an A of 1.0 and 0.8, respectively. If $Hazardous\ Function\ Sequence_1$ has adherence metadata and $Hazardous\ Function\ Sequence_2$ has both adherence and invasion metadata, the fingerprint score contribution for CDS_i would be 1.0 for adherence and 0.8 for invasion. Maximum A scores for each functional category for each strain were then summed across each strain's CDSs. The final fingerprint score for each strain was defined as the cumulative A within each category normalized by the strain's total number of CDSs then normalized by the maximal value across all pathogen and nonpathogen strains within that functional category.

Hierarchical clustering analysis was performed in R using the function hclust, with UPGMA as the method for agglomerative clustering. Dendrograms were plotted using the R libraries ggdendro and ggplot2.

Authors note

The authors have carefully reviewed and discussed the concepts in this manuscript for dual use concerns both internally as well as with members of the US Government, the International Gene Synthesis Consortium (IGSC), and Engineering Research Council (EBRC). While we understand the risks, the prevailing opinion is that the methodologies presented here in themselves do not provide a roadmap for creation of harmful organisms, nor do they enable circumvention of screening. In fact, this manuscript provides the scientific community a potential framework for screening, which should help improve biosecurity through improved screening practices. Further, the authors purposefully did not publicize our database to further alleviate such concerns.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Author contributions

CB contributed to the conceptualization of the paper and writing. BG contributed to curation, writing, and analysis. OT contributed to conceptualization and review. CM contributed to analysis and review. CH, DH, ZS, and LH contributed to data curation.

Funding

This work supported in part by the Office of the Director of National Intelligence (ODNI), Intelligence Advanced Research Projects Activity (IARPA), *via* contract number W911NF-17-C-0052. The views and conclusions contained herein are those of the authors and should not be interpreted as necessarily representing the official policies or endorsements, either expressed or implied, of the ODNI, IARPA, or the U.S. Government. The U.S. Government is authorized to reproduce and distribute reprints for Governmental purposes notwithstanding any copyright annotation thereon.

Acknowledgments

A special thanks to Gene Godbold, Sara Nitcher, Rachel Spurbeck, Meg Howard, Morris Makobongo, Nikolas Kanel, David Eaton, and Brett Fowle for their contributions to populating information into the biological functions hazard database and developing software for automated hazard level predictions based on protein metadata.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer RM declared a shared research group with the author CB to the handling editor.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2022.979497/full#supplementary-material

Hazardous Functions Partially Separate *E. coli* Pathogen Groups Shown are the dendrograms for *E. coli* grouped by type of *E. coli*. Pathogenic species colored as follows: EHEC (red), ExPec/UPEC (purple), EAEC/ETEC/AIEC/EPEC (orange). Non-pathogenic species are colored as follows: commensal (green and teal) and yellow (lab strains).

References

Abdulamir, A. S., Jassim, S. A., and Abu Bakar, F. (2014). Novel approach of using a cocktail of designed bacteriophages against gut pathogenic *E. coli* for bacterial load biocontrol. *Ann. Clin. Microbiol. Antimicrob.* 13, 39. doi:10.1186/s12941-014-0039-z

Adimpong, D. B., Sorensen, K. I., Thorsen, L., Stuer-Lauridsen, B., Abdelgadir, W. S., Nielsen, D. S., et al. (2012). Antimicrobial susceptibility of Bacillus strains isolated from primary starters for African traditional bread production and characterization of the bacitracin operon and bacitracin biosynthesis. *Appl. Environ. Microbiol.* 78, 7903–7914. doi:10.1128/aem.00730-12

Agarwal, N., Lamichhane, G., Gupta, R., Nolan, S., and Bishai, W. R. (2009). Cyclic AMP intoxication of macrophages by a *Mycobacterium tuberculosis* adenylate cyclase. *Nature* 460, 98–102. doi:10.1038/nature08123

Ahr, B., Robert-Hebmann, V., Devaux, C., and Biard-Piechaczyk, M. (2004). Apoptosis of uninfected cells induced by HIV envelope glycoproteins. *Retrovirology* 1, 12. doi:10.1186/1742-4690-1-12

Aldrich, J. V., and McLaughlin, J. P. (2012). Opioid peptides: Potential for drug development. *Drug Discov. Today Technol.* 9, e23–e31. doi:10.1016/j.ddtec.2011.07.007

Al-Tebrineh, J., Mihali, T. K., Pomati, F., and Neilan, B. A. (2010). Detection of saxitoxin-producing cyanobacteria and Anabaena circinalis in environmental water blooms by quantitative PCR. *Appl. Environ. Microbiol.* 76, 7836–7842. doi:10.1128/aem.00174-10

Andersson, D. I., and Hughes, D. (2010). Antibiotic resistance and its cost: Is it possible to reverse resistance? *Nat. Rev. Microbiol.* 8, 260–271. doi:10.1038/nrmicro2319

Andreevskaia, S. N., Chernousova, L. N., Smirnova, T. G., Larionova, E. E., and Kuz'min, A. V. (2006). *Mycobacterium tuberculosis* strain transmission caused by migratory processes in the Russian Federation (in case of populational migration from the Caucasian Region to Moscow and the Moscow Region). *Probl. Tuberk. Bolezn. Legk.* 1. 29–35.

Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., et al. (2000). Gene ontology: Tool for the unification of biology. *Nat. Genet.* 25, 25–29. doi:10.1038/75556

Ashida, H., Mimuro, H., Ogawa, M., Kobayashi, T., Sanada, T., Kim, M., et al. (2011). Cell death and infection: A double-edged sword for host and pathogen survival. *J. Cell Biol.* 195, 931–942. doi:10.1083/jcb.201108081

Awad, M. M., Johanesen, P. A., Carter, G. P., Rose, E., and Lyras, D. (2014). *Clostridium difficile* virulence factors: Insights into an anaerobic spore-forming pathogen. *Gut Microbes* 5, 579–593. doi:10.4161/19490976.2014.969632

Ayyavoo, V., Mahboubi, A., Mahalingam, S., Ramalingam, R., Kudchodkar, S., Williams, W. V., et al. (1997). HIV-1 Vpr suppresses immune activation and apoptosis through regulation of nuclear factor κB. *Nat. Med.* 3, 1117–1123. doi:10.1038/nm1097-1117

Bakour, S., Sankar, S. A., Rathored, J., Biagini, P., Raoult, D., and Fournier, P. E. (2016). Identification of virulence factors and antibiotic resistance markers using bacterial genomics. *Future Microbiol.* 11, 455–466. doi:10.2217/fmb.15.149

Barth, H., Aktories, K., Popoff, M. R., and Stiles, B. G. (2004). Binary bacterial toxins: Biochemistry, biology, and applications of common Clostridium and Bacillus proteins. *Microbiol. Mol. Biol. Rev.* 68, 373–402. doi:10.1128/mmbr.68.3.373-402.2004

Bartra, S. S., Styer, K. L., O'Bryant, D. M., Nilles, M. L., Hinnebusch, B. J., Aballay, A., et al. (2008). Resistance of *Yersinia pestis* to complement-dependent killing is mediated by the Ail outer membrane protein. *Infect. Immun.* 76, 612–622. doi:10.1128/iai.01125-07

Basso, P., Ragno, M., Elsen, S., Reboud, E., Golovkine, G., Bouillot, S., et al. (2017). *Pseudomonas aeruginosa* pore-forming exolysin and type IV pili cooperate to induce host cell lysis. *MBio* 8, e02250–16. doi:10.1128/mbio.02250-16

Benfield, A. P., Goodey, N. M., Phillips, L. T., and Martin, S. F. (2007). Structural studies examining the substrate specificity profiles of PC-PLC(Bc) protein variants. *Arch. Biochem. Biophys.* 460, 41–47. doi:10.1016/j.abb.2007.01.023

Bernard, S. C., Simpson, N., Join-Lambert, O., Federici, C., Laran-Chich, M. P., Maissa, N., et al. (2014). Pathogenic Neisseria meningitidis utilizes CD147 for vascular colonization. *Nat. Med.* 20, 725–731. doi:10.1038/nm.3563

Blair, J. M., Webber, M. A., Baylay, A. J., Ogbolu, D. O., and Piddock, L. J. (2015). Molecular mechanisms of antibiotic resistance. *Nat. Rev. Microbiol.* 13, 42–51. doi:10.1038/nrmicro3380

Blight, K. J., Kolykhalov, A. A., and Rice, C. M. (2000). Efficient initiation of HCV RNA replication in cell culture. *Science* 290, 1972–1974. doi:10.1126/science.290.5498.1972

Borzenkov, V. M., Pomerantsev, A. P., and Ashmarin, I. P. (1993). The additive synthesis of a regulatory peptide *in vivo*: The administration of a vaccinal francisella tularensis strain that produces beta-endorphin. *Biull. Eksp. Biol. Med.* 116, 151–153.

Borzenkov, V. M., Pomerantsev, A. P., Pomerantseva, O. M., and Ashmarin, I. P. (1994). Study of nonpathogenic strains of francisella, brucella and yersinia as producers of recombinant beta-endorphin. *Biull. Eksp. Biol. Med.* 117, 612–615.

Brbic, M., Piskorec, M., Vidulin, V., Krisko, A., Smuc, T., and Supek, F. (2016). The landscape of microbial phenotypic traits and associated genes. *Nucleic Acids Res.* 44, 10074–10090. doi:10.1093/nar/gkw964

Burns, D. (2003). *Bacterial protein toxins*. Washington, D.C. ASM Press.

Campos, A., and Vasconcelos, V. (2010). Molecular mechanisms of microcystin toxicity in animal cells. *Int. J. Mol. Sci.* 11, 268–287. doi:10.3390/ijms11010268

Casadevall, A., and Pirofski, L. A. (2003). The damage-response framework of microbial pathogenesis. *Nat. Rev. Microbiol.* 1, 17–24. doi:10.1038/nrmicro732

Casilag, F., Lorenz, A., Krueger, J., Klawonn, F., Weiss, S., and Haussler, S. (2016). The LasB elastase of *Pseudomonas aeruginosa* acts in concert with alkaline protease AprA to prevent flagellin-mediated immune recognition. *Infect. Immun.* 84, 162–171. doi:10.1128/iai.00939-15

Caspi, R., Billington, R., Fulcher, C. A., Keseler, I. M., Kothari, A., Krummenacker, M., et al. (2018). The MetaCyc database of metabolic pathways and enzymes. *Nucleic Acids Res.* 46, D633–D639. doi:10.1093/nar/gkx935

Cello, J., Paul, A. V., and Wimmer, E. (2002). Chemical synthesis of poliovirus cDNA: Generation of infectious virus in the absence of natural template. *Science* 297, 1016–1018. doi:10.1126/science.1072266

Chambers, H. F. (1997). Methicillin resistance in staphylococci: Molecular and biochemical basis and clinical implications. *Clin. Microbiol. Rev.* 10, 781–791. doi:10.1128/cmr.10.4.781

Chapman, D. A., Tcherepanov, V., Upton, C., and Dixon, L. K. (2008). Comparison of the genome sequences of non-pathogenic and pathogenic African swine fever virus isolates. *J. Gen. Virol.* 89, 397–408. doi:10.1099/vir.0.83343-0

Chen, Z., Franco, C. F., Baptista, R. P., Cabral, J. M., Coelho, A. V., Rodrigues, C. J., Jr., et al. (2007). Purification and identification of cutinases from Colletotrichum kahawae and Colletotrichum gloeosporioides. *Appl. Microbiol. Biotechnol.* 73, 1306–1313. doi:10.1007/s00253-006-0605-1

Chen, N., Bellone, C. J., Schriewer, J., Owens, G., Fredrickson, T., Parker, S., et al. (2011). Poxvirus interleukin-4 expression overcomes inherent resistance and vaccine-induced immunity: Pathogenesis, prophylaxis, and antiviral therapy. *Virology* 409, 328–337. doi:10.1016/j.virol.2010.10.021

Colf, L. A. (2016). Preparing for nontraditional biothreats. *Health Secur.* 14, 7–12. doi:10.1089/hs.2015.0045

Cook, J. D., and Lee, J. E. (2013). The secret life of viral entry glycoproteins: Moonlighting in immune evasion. *PLoS Pathog.* 9, e1003258. doi:10.1371/journal.ppat.1003258

Cornelis, G. R. (2000). Molecular and cell biology aspects of plague. *Proc. Natl. Acad. Sci. U. S. A.* 97, 8778–8783. doi:10.1073/pnas.97.16.8778

Cosentino, S., Voldby Larsen, M., Moller Aarestrup, F., and Lund, O. (2013). PathogenFinder--distinguishing friend from foe using bacterial whole genome sequence data. *PLoS One* 8, e77302. doi:10.1371/journal.pone.0077302

Dean, R. A., Talbot, N. J., Ebbole, D. J., Farman, M. L., Mitchell, T. K., Orbach, M. J., et al. (2005). The genome sequence of the rice blast fungus Magnaporthe grisea. *Nature* 434, 980–986. doi:10.1038/nature03449

Dickers, K. J., Bradberry, S. M., Rice, P., Griffiths, G. D., and Vale, J. A. (2003). Abrin poisoning. *Toxicol. Rev.* 22, 137–142. doi:10.2165/00139709-200322030-00002

Dobrindt, U. (2005). (Patho-)Genomics of *Escherichia coli*. *Int. J. Med. Microbiol.* 295, 357–371. doi:10.1016/j.ijmm.2005.07.009

Dobson, C. M. (2001). The structural basis of protein folding and its links with human disease. *Phil. Trans. R. Soc. Lond. B* 356, 133–145. doi:10.1098/rstb.2000.0758

Dudak, F. C., Boyaci, I. H., and Orner, B. P. (2011). The discovery of small-molecule mimicking peptides through phage display. *Molecules* 16, 774–789. doi:10.3390/molecules16010774

Dulon, S., Leduc, D., Cottrell, G. S., D'Alayer, J., Hansen, K. K., Bunnett, N. W., et al. (2005). *Pseudomonas aeruginosa* elastase disables proteinase-activated receptor 2 in respiratory epithelial cells. *Am. J. Respir. Cell Mol. Biol.* 32, 411–419. doi:10.1165/rcmb.2004-0274oc

Ebrahimi, M., Aghagolzadeh, P., Shamabadi, N., Tahmasebi, A., Alsharifi, M., Adelson, D. L., et al. (2014). Understanding the underlying mechanism of HA-subtyping in the level of physic-chemical characteristics of protein. *PLoS One* 9, e96984. doi:10.1371/journal.pone.0096984

EMBL-EBI (2019). Toxin activity. Available at: https://www.ebi.ac.uk/QuickGO/term/GO:0090729 (Accessed November 18, 2019).

Erana, H., and Castilla, J. (2016). The architecture of prions: How understanding would provide new therapeutic insights. *Swiss Med. Wkly.* 146, w14354. doi:10.4414/smw.2016.14354

Espinosa Angarica, V., Angulo, A., Giner, A., Losilla, G., Ventura, S., and Sancho, J. (2014). PrionScan: An online database of predicted prion domains in complete proteomes. *BMC Genomics* 15, 102. doi:10.1186/1471-2164-15-102

Esvelt, K. M., Smidler, A. L., Catteruccia, F., and Church, G. M. (2014). Concerning RNA-guided gene drives for the alteration of wild populations. *Elife* 3, e03401. doi:10.7554/elife.03401

Federal Registar (2022). Screening framework guidance for providers and users of synthetic oligonucleotides. Available at: https://www.federalregister.gov/documents/2022/04/29/2022-09210/screening-framework-guidance-for-providers-and-users-of-synthetic-oligonucleotides.

Finlay, B. B. (2005). Bacterial virulence strategies that utilize Rho GTPases. *Curr. Top. Microbiol. Immunol.* 291, 1–10. doi:10.1007/3-540-27511-8_1

Flores-Diaz, M., and Alape-Giron, A. (2003). Role of *Clostridium perfringens* phospholipase C in the pathogenesis of gas gangrene. *Toxicon* 42, 979–986. doi:10.1016/j.toxicon.2003.11.013

Forrellad, M. A., Klepp, L. I., Gioffre, A., Sabio y Garcia, J., Morbidoni, H. R., de la Paz Santangelo, M., et al. (2013). Virulence factors of the *Mycobacterium tuberculosis* complex. *Virulence* 4, 3–66. doi:10.4161/viru.22329

Fournier, P. E., Richet, H., and Weinstein, R. A. (2006). The epidemiology and control of Acinetobacter baumannii in health care facilities. *Clin. Infect. Dis.* 42, 692–699. doi:10.1086/500202

Francica, J. R., Varela-Rohena, A., Medvec, A., Plesa, G., Riley, J. L., and Bates, P. (2010). Steric shielding of surface epitopes and impaired immune recognition induced by the ebola virus glycoprotein. *PLoS Pathog.* 6, e1001098. doi:10.1371/journal.ppat.1001098

Francis, J. W., Brown, R. H., Jr., Figueiredo, D., Remington, M. P., Castillo, O., Schwarzschild, M. A., et al. (2000). Enhancement of diphtheria toxin potency by replacement of the receptor binding domain with tetanus toxin C-fragment: A potential vector for delivering heterologous proteins to neurons. *J. Neurochem.* 74, 2528–2536. doi:10.1046/j.1471-4159.2000.0742528.x

Friebe, S., van der Goot, F. G., and Burgi, J. (2016). The ins and outs of anthrax toxin. *Toxins (Basel)* 8, 69. doi:10.3390/toxins8030069

Frolkis, A., Knox, C., Lim, E., Jewison, T., Law, V., Hau, D. D., et al. (2010). Smpdb: The small molecule pathway database. *Nucleic Acids Res.* 38, D480–D487. doi:10.1093/nar/gkp1002

Galanie, S., Thodey, K., Trenchard, I. J., Filsinger Interrante, M., and Smolke, C. D. (2015). Complete biosynthesis of opioids in yeast. *Science* 349, 1095–1100. doi:10.1126/science.aac9373

Galyov, E. E., Brett, P. J., and DeShazer, D. (2010). Molecular insights into Burkholderia pseudomallei and Burkholderia mallei pathogenesis. *Annu. Rev. Microbiol.* 64, 495–517. doi:10.1146/annurev.micro.112408.134030

Gautam, A., Chaudhary, K., Singh, S., Joshi, A., Anand, P., Tuknait, A., et al. (2014). Hemolytik: A database of experimentally determined hemolytic and non-hemolytic peptides. *Nucleic Acids Res.* 42, D444–D449. doi:10.1093/nar/gkt1008

Geisinger, E., and Isberg, R. R. (2017). Interplay between antibiotic resistance and virulence during disease promoted by multidrug-resistant bacteria. *J. Infect. Dis.* 215, S9–S17. doi:10.1093/infdis/jiw402

Gibson, D. G., Glass, J. I., Lartigue, C., Noskov, V. N., Chuang, R. Y., Algire, M. A., et al. (2010). Creation of a bacterial cell controlled by a chemically synthesized genome. *Science* 329, 52–56. doi:10.1126/science.1190719

Gilmour, M. W., Graham, M., Reimer, A., and Van Domselaar, G. (2013). Public health genomics and the new molecular epidemiology of bacterial pathogens. *Public Health Genomics* 16, 25–30. doi:10.1159/000342709

Godbold Gd, K. A., LeSassier, D. S., Treangen, T. J., and Ternus, K. L. (2021). Categorizing sequences of concern by function to better assess mechanisms of microbial pathogenesis. *Infect. Immun.* 90, e0033421. doi:10.1128/iai.00334-21

Gold, J. A., Hoshino, Y., Jones, M. B., Hoshino, S., Nolan, A., and Weiden, M. D. (2007). Exogenous interferon-alpha and interferon-gamma increase lethality of murine inhalational anthrax. *PLoS One* 2, e736. doi:10.1371/journal.pone.0000736

Goldberg, J. B. (2010). Why is *Pseudomonas aeruginosa* a pathogen? F1000. *F1000 Biol. Rep.* 2, 29. doi:10.3410/b2-29

Goldman, A. S. (2000). Back to basics: Host responses to infection. *Pediatr. Rev.* 21, 342–349. doi:10.1542/pir.21.10.342

Green, E. R., and Mecsas, J. (2016). Bacterial secretion systems: An overview. *Microbiol. Spectr.* 4, 1–32. doi:10.1128/microbiolspec.vmbf-0012-2015

Haas, B., and Grenier, D. (2018). Understanding the virulence of Streptococcus suis: A veterinary, medical, and economic challenge. *Med. Maladies Infect.* 48, 159–166. doi:10.1016/j.medmal.2017.10.001

Hamada, S., Kawabata, S., and Nakagawa, I. (2015). Molecular and genomic characterization of pathogenic traits of group A Streptococcus pyogenes. *Proc. Jpn. Acad. Ser. B. Phys. Biol. Sci.* 91, 539–559. doi:10.2183/pjab.91.539

Harbi, D., Parthiban, M., Gendoo, D. M., Ehsani, S., Kumar, M., Schmitt-Ulms, G., et al. (2012). PrionHome: A database of prions and other sequences relevant to prion phenomena. *PLoS One* 7, e31785. doi:10.1371/journal.pone.0031785

Haschek, W., and Voss, K. (2013). *Rousseaux's handbook of toxicologic pathology*. Amsterdam, Netherlands: Elsevier.

Hauswedell, H., Singer, J., and Reinert, K. (2014). Lambda: The local aligner for massive biological data. *Bioinformatics* 30, i349–55. doi:10.1093/bioinformatics/btu439

Headquarters Department of the Army (2018) Nuclear and chemical weapons and materiel chemical surety. Available at: https://armypubs.army.mil/epubs/DR_pubs/DR_a/pdf/web/ARN3125_AR50-6_WEB_FINAL.pdf (Accessed April 16, 2018).

Herfst, S., Schrauwen, E. J., Linster, M., Chutinimitkul, S., de Wit, E., Munster, V. J., et al. (2012). Airborne transmission of influenza A/H5N1 virus between ferrets. *Science* 336, 1534–1541. doi:10.1126/science.1213362

Hermjakob, H., Montecchi-Palazzi, L., Lewington, C., Mudali, S., Kerrien, S., Orchard, S., et al. (2004). IntAct: An open source molecular interaction database. *Nucleic Acids Res.* 32, D452–D455. doi:10.1093/nar/gkh052

Herpfer, I., Katzev, M., Feige, B., Fiebich, B. L., Voderholzer, U., and Lieb, K. (2007). Effects of substance P on memory and mood in healthy male subjects. *Hum. Psychopharmacol. Clin. Exp.* 22, 567–573. doi:10.1002/hup.876

Ho, N. K., Henry, A. C., Johnson-Henry, K., and Sherman, P. M. (2013). Pathogenicity, host responses and implications for management of enterohemorrhagic *Escherichia coli* O157:H7 infection. *Can. J. Gastroenterology* 27, 281–285. doi:10.1155/2013/138673

Holden, M. T., Titball, R. W., Peacock, S. J., Cerdeno-Tarraga, A. M., Atkins, T., Crossman, L. C., et al. (2004). Genomic plasticity of the causative agent of melioidosis, Burkholderia pseudomallei. *Proc. Natl. Acad. Sci. U. S. A.* 101, 14240–14245. doi:10.1073/pnas.0403302101

Hu, P., Yang, M., Zhang, A., Wu, J., Chen, B., Hua, Y., et al. (2011). Complete genome sequence of Streptococcus suis serotype 3 strain ST3. *J. Bacteriol.* 193, 3428–3429. doi:10.1128/jb.05018-11

Huang, W. J., Chen, W. W., and Zhang, X. (2015). Prions mediated neurodegenerative disorders. *Eur. Rev. Med. Pharmacol. Sci.* 19, 4028–4034.

Hudson, C. M., Lau, B. Y., and Williams, K. P. (2015). Islander: A database of precisely mapped genomic islands in tRNA and tmRNA genes. *Nucleic Acids Res.* 43, D48–D53. doi:10.1093/nar/gku1072

Hulo, C., de Castro, E., Masson, P., Bougueleret, L., Bairoch, A., Xenarios, I., et al. (2011). ViralZone: A knowledge resource to understand virus diversity. *Nucleic Acids Res.* 39, D576–D582. doi:10.1093/nar/gkq901

Hutchison, C. A., 3rd, Chuang, R. Y., Noskov, V. N., Assad-Garcia, N., Deerinck, T. J., Ellisman, M. H., et al. (2016). Design and synthesis of a minimal bacterial genome. *Science* 351, aad6253. doi:10.1126/science.aad6253

Hwang, I. Y., Koh, E., Wong, A., March, J. C., Bentley, W. E., Lee, Y. S., et al. (2017). Engineered probiotic *Escherichia coli* can eliminate and prevent *Pseudomonas aeruginosa* gut infection in animal models. *Nat. Commun.* 8, 15028. doi:10.1038/ncomms15028

IARPA. Broad Agency Announcement (2016). Functional genomic and computational assessment of threats (fun GCAT). IARPA-BAA-16-08. Available at: https://viterbi.usc.edu/links/webuploads/Functional%20Genomic%20and%20Computational%20Assessment%20of%20Threats%20(Fun%20GCAT)%20IARPA-BAA-16-08.pdf.

Ilina, E. N., Shitikov, E. A., Ikryannikova, L. N., Alekseev, D. G., Kamashev, D. E., Malakhova, M. V., et al. (2013). Comparative genomic analysis of *Mycobacterium tuberculosis* drug resistant strains from Russia. *PLoS One* 8, e56577. doi:10.1371/journal.pone.0056577

Inoshima, I., Inoshima, N., Wilke, G. A., Powers, M. E., Frank, K. M., Wang, Y., et al. (2011). A *Staphylococcus aureus* pore-forming toxin subverts the activity of ADAM10 to cause lethal infection in mice. *Nat. Med.* 17, 1310–1314. doi:10.1038/nm.2451

International Gene Synthesis Consortium (2017) Harmonized screening protocol v2.0 gene sequence & customer screening to promote biosecurity. Available at: https://genesynthesisconsortium.org/wp-content/uploads/IGSCHarmonizedProtocol11-21-17.pdf (Accessed November 19, 2017).

Ireton, K. (2013). Molecular mechanisms of cell-cell spread of intracellular bacterial pathogens. *Open Biol.* 3, 130079. doi:10.1098/rsob.130079

Izard, T., Tran Van Nhieu, G., and Bois, P. R. (2006). Shigella applies molecular mimicry to subvert vinculin and invade host cells. *J. Cell Biol.* 175, 465–475. doi:10.1083/jcb.200605091

Jackson, R. J., Ramsay, A. J., Christensen, C. D., Beaton, S., Hall, D. F., and Ramshaw, I. A. (2001). Expression of mouse interleukin-4 by a recombinant ectromelia virus suppresses cytolytic lymphocyte responses and overcomes genetic resistance to mousepox. *J. Virol.* 75, 1205–1210. doi:10.1128/jvi.75.3.1205-1210.2001

Jacoby, G. A., and Munoz-Price, L. S. (2005). The new beta-lactamases. *N. Engl. J. Med. Overseas. Ed.* 352, 380–391. doi:10.1056/nejmra041359

Jal, S., and Khora, S. S. (2015). An overview on the origin and production of tetrodotoxin, a potent neurotoxin. *J. Appl. Microbiol.* 119, 907–916. doi:10.1111/jam.12896

Jia, B., Raphenya, A. R., Alcock, B., Waglechner, N., Guo, P., Tsang, K. K., et al. (2017). Card 2017: Expansion and model-centric curation of the comprehensive antibiotic resistance database. *Nucleic Acids Res.* 45, D566–D573. doi:10.1093/nar/gkw1004

Jirschitzka, J., Schmidt, G. W., Reichelt, M., Schneider, B., Gershenzon, J., and D'Auria, J. C. (2012). Plant tropane alkaloid biosynthesis evolved independently in the Solanaceae and Erythroxylaceae. *Proc. Natl. Acad. Sci. U. S. A.* 109, 10304–10309. doi:10.1073/pnas.1200473109

Jorgensen, R., Purdy, A. E., Fieldhouse, R. J., Kimber, M. S., Bartlett, D. H., and Merrill, A. R. (2008). Cholix toxin, a novel ADP-ribosylating factor from *Vibrio cholerae*. *J. Biol. Chem.* 283, 10671–10678. doi:10.1074/jbc.m710008200

Joshi-Tope, G., Gillespie, M., Vastrik, I., D'Eustachio, P., Schmidt, E., de Bono, B., et al. (2005). Reactome: A knowledgebase of biological pathways. *Nucleic Acids Res.* 33, D428–D432. doi:10.1093/nar/gki072

Jungo, F., Bougueleret, L., Xenarios, I., and Poux, S. (2012). The UniProtKB/Swiss-prot tox-prot program: A central hub of integrated venom protein data. *Toxicon* 60, 551–557. doi:10.1016/j.toxicon.2012.03.010

Kastin, A. (2013). *Handbook of biologically active peptides*. Amsterdam, Netherlands: Elsevier.

Kaur, P., Chakraborti, A., and Asea, A. (2010). Enteroaggregative *Escherichia coli*: An emerging enteric food borne pathogen. *Interdiscip. Perspect. Infect. Dis.* 2010, 1–10. doi:10.1155/2010/254159

Kempf, M., and Rolain, J. M. (2012). Emergence of resistance to carbapenems in acinetobacter baumannii in europe: Clinical impact and therapeutic options. *Int. J. Antimicrob. Agents* 39, 105–114. doi:10.1016/j.ijantimicag.2011.10.004

Kerr, P. J., Perkins, H. D., Inglis, B., Stagg, R., McLaughlin, E., Collins, S. V., et al. (2004). Expression of rabbit IL-4 by recombinant myxoma viruses enhances virulence and overcomes genetic resistance to myxomatosis. *Virology* 324, 117–128. doi:10.1016/j.virol.2004.02.031

Khairil Anuar, I. N. A., Banerjee, A., Keeble, A. H., Carella, A., Nikov, G. I., and Howarth, M. (2019). Spy&Go purification of SpyTag-proteins using pseudo-SpyCatcher to access an oligomerization toolbox. *Nat. Commun.* 10, 1734. doi:10.1038/s41467-019-09678-w

Kiessling, K. (1986). Biochemical mechanism of action of mycotoxins. *Pure Appl. Chem.* 58, 327–338. doi:10.1351/pac198658020327

Klein, A. M., Vaissiere, B. E., Cane, J. H., Steffan-Dewenter, I., Cunningham, S. A., Kremen, C., et al. (2007). Importance of pollinators in changing landscapes for world crops. *Proc. R. Soc. B* 274, 303–313. doi:10.1098/rspb.2006.3721

Knodler, L. A., Celli, J., and Finlay, B. B. (2001). Pathogenic trickery: Deception of host cell processes. *Nat. Rev. Mol. Cell Biol.* 2, 578–588. doi:10.1038/35085062

Kohler, C. D., and Dobrindt, U. (2011). What defines extraintestinal pathogenic *Escherichia coli*? *Int. J. Med. Microbiol.* 301, 642–647. doi:10.1016/j.ijmm.2011.09.006

Korbsrisate, S., Tomaras, A. P., Damnin, S., Ckumdee, J., Srinon, V., Lengwehasatit, I., et al. (2007). Characterization of two distinct phospholipase C enzymes from Burkholderia pseudomallei. *Microbiology* 153, 1907–1915. doi:10.1099/mic.0.2006/003004-0

Kurupati, P., Turner, C. E., Tziona, I., Lawrenson, R. A., Alam, F. M., Nohadani, M., et al. (2010). Chemokine-cleaving Streptococcus pyogenes protease SpyCEP is necessary and sufficient for bacterial dissemination within soft tissues and the respiratory tract. *Mol. Microbiol.* 76, 1387–1397. doi:10.1111/j.1365-2958.2010.07065.x

Kuzmenkov, A. I., Krylov, N. A., Chugunov, A. O., Grishin, E. V., and Vassilevski, A. A. (2016). Kalium: A database of potassium channel toxins from scorpion venom. *Database (Oxford)* 2016, baw056. doi:10.1093/database/baw056

Lago, J., Rodriguez, L. P., Blanco, L., Vieites, J. M., and Cabado, A. G. (2015). Tetrodotoxin, an extremely potent marine neurotoxin: Distribution, toxicity, origin and therapeutical uses. *Mar. Drugs* 13, 6384–6406. doi:10.3390/md13106384

Lamkanfi, M., and Dixit, V. M. (2010). Manipulation of host cell death pathways during microbial infections. *Cell Host Microbe* 8, 44–54. doi:10.1016/j.chom.2010.06.007

Legname, G., Baskakov, I. V., Nguyen, H. O., Riesner, D., Cohen, F. E., DeArmond, S. J., et al. (2004). Synthetic mammalian prions. *Science* 305, 673–676. doi:10.1126/science.1100195

Lewis, R., Dutertre, S., Vetter, I., and Christie, M. (2012). Conus venom peptide pharmacology. *Pharmacol. Rev.* 64, 259–298. doi:10.1124/pr.111.005322

Li, Q., Zhang, C., Chen, H., Xue, J., Guo, X., Liang, M., et al. (2018). BioPepDB: An integrated data platform for food-derived bioactive peptides. *Int. J. Food Sci. Nutr.* 69, 963–968. doi:10.1080/09637486.2018.1446916

Liu, B., Zheng, D., Jin, Q., Chen, L., and Yang, J. (2019). Vfdb 2019: A comparative pathogenomic platform with an interactive web interface. *Nucleic Acids Res.* 47, D687–D692. doi:10.1093/nar/gky1080

Liu, M., Li, X., Xie, Y., Bi, D., Sun, J., Li, J., et al. (2019). ICEberg 2.0: An updated database of bacterial integrative and conjugative elements. *Nucleic Acids Res.* 47, D660–D665. doi:10.1093/nar/gky1123

Lu, T., Yao, B., and Zhang, C. (2012). Dfvf: Database of fungal virulence factors. *Database (Oxford)*, 2012 bas032. doi:10.1093/database/bas032

Lu, Q. F., Cao, D. M., Su, L. L., Li, S. B., Ye, G. B., Zhu, X. Y., et al. (2019). Genus-wide comparative genomics analysis of Neisseria to identify new genes associated with pathogenicity and niche adaptation of Neisseria pathogens. *Int. J. Genomics* 2019, 1–19. doi:10.1155/2019/6015730

Luo, N., Pereira, S., Sahin, O., Lin, J., Huang, S., Michel, L., et al. (2005). Enhanced *in vivo* fitness of fluoroquinolone-resistant Campylobacter jejuni in the absence of antibiotic selection pressure. *Proc. Natl. Acad. Sci. U. S. A.* 102, 541–546. doi:10.1073/pnas.0408966102

Luo, G., Ibrahim, A. S., Spellberg, B., Nobile, C. J., Mitchell, A. P., and Fu, Y. (2010). Candida albicans Hyr1p confers resistance to neutrophil killing and is a potential vaccine target. *J. Infect. Dis.* 201, 1718–1728. doi:10.1086/652407

Magarlamov, T. Y., Melnikova, D. I., and Chernyshev, A. V. (2017). Tetrodotoxin-producing bacteria: Detection, distribution and migration of the toxin in aquatic systems. *Toxins (Basel)* 9, 166. doi:10.3390/toxins9050166

Mahenthiralingam, E., and Vandamme, P. (2005). Taxonomy and pathogenesis of the Burkholderia cepacia complex. *Chron. Respir. Dis.* 2, 209–217. doi:10.1191/1479972305cd053ra

Mathur, D., Prakash, S., Anand, P., Kaur, H., Agrawal, P., Mehta, A., et al. (2016). PEPlife: A repository of the half-life of peptides. *Sci. Rep.* 6, 36617. doi:10.1038/srep36617

Memisevic, V., Kumar, K., Cheng, L., Zavaljevski, N., DeShazer, D., Wallqvist, A., et al. (2014). DBSecSys: A database of Burkholderia mallei secretion systems. *BMC Bioinforma.* 15, 244. doi:10.1186/1471-2105-15-244

Mihara, T., Nishimura, Y., Shimizu, Y., Nishiyama, H., Yoshikawa, G., Uehara, H., et al. (2016). Linking virus genomes with host taxonomy. *Viruses* 8, 66. doi:10.3390/v8030066

Mitchell, A. M., and Mitchell, T. J. (2010). Streptococcus pneumoniae: Virulence factors and variation. *Clin. Microbiol. Infect.* 16, 411–418. doi:10.1111/j.1469-0691.2010.03183.x

Mitchell, A. L., Attwood, T. K., Babbitt, P. C., Blum, M., Bork, P., Bridge, A., et al. (2019). InterPro in 2019: Improving coverage, classification and access to protein sequence annotations. *Nucleic Acids Res.* 47, D351–D360. doi:10.1093/nar/gky1100

Mueller, M., Grauschopf, U., Maier, T., Glockshuber, R., and Ban, N. (2009). The structure of a cytolytic alpha-helical toxin pore reveals its assembly mechanism. *Nature* 459, 726–730. doi:10.1038/nature08026

Nakagawa, A., Matsumura, E., Koyanagi, T., Katayama, T., Kawano, N., Yoshimatsu, K., et al. (2016). Total biosynthesis of opiates by stepwise fermentation using engineered *Escherichia coli*. *Nat. Commun.* 7, 10390. doi:10.1038/ncomms10390

National Cancer Institute (2019). Aflatoxins. Available at: https://www.cancer.gov/about-cancer/causes-prevention/risk/substances/aflatoxins (Accessed October 10, 2019).

NCBI (2019). Batch Entrez. Available at: https://www.ncbi.nlm.nih.gov/sites/batchentrez (Accessed November 18, 2019).

Nesic, D., and Stebbins, C. E. (2005). Mechanisms of assembly and cellular interactions for the bacterial genotoxin CDT. *PLoS Pathog.* 1, e28. doi:10.1371/journal.ppat.0010028

Newby, D. E., Sciberras, D. G., Ferro, C. J., Gertz, B. J., Sommerville, D., Majumdar, A., et al. (1999). Substance P-induced vasodilatation is mediated by the neurokinin type 1 receptor but does not contribute to basal vascular tone in man. *Br. J. Clin. Pharmacol.* 48, 336–344. doi:10.1046/j.1365-2125.1999.00017.x

Nguyen, Y., and Sperandio, V. (2012). Enterohemorrhagic *E. coli* (EHEC) pathogenesis. *Front. Cell. Infect. Microbiol.* 2, 90. doi:10.3389/fcimb.2012.00090

Nielsen, S. D., Beverly, R. L., Qu, Y., and Dallas, D. C. (2017). Milk bioactive peptide database: A comprehensive database of milk protein-derived bioactive peptides and novel visualization. *Food Chem. x.* 232, 673–682. doi:10.1016/j.foodchem.2017.04.056

Niu, C., Yu, D., Wang, Y., Ren, H., Jin, Y., Zhou, W., et al. (2013). Common and pathogen-specific virulence factors are different in function and structure. *Virulence* 4, 473–482. doi:10.4161/viru.25730

Noor Uddin, G. M., Larsen, M. H., Christensen, H., Aarestrup, F. M., Phu, T. M., and Dalsgaard, A. (2015). Identification and antimicrobial resistance of bacteria isolated from probiotic products used in shrimp culture. *PLoS One* 10, e0132338. doi:10.1371/journal.pone.0132338

Noyce, R. S., Lederman, S., and Evans, D. H. (2018). Construction of an infectious horsepox virus vaccine from chemically synthesized DNA fragments. *PLoS One* 13, e0188453. doi:10.1371/journal.pone.0188453

Nuermberger, E. L., and Bishai, W. R. (2004). Antibiotic resistance in Streptococcus pneumoniae: What does the future hold? *Clin. Infect. Dis.* 38 (4), S363–S371. doi:10.1086/382696

O'Brien, A. D., Tesh, V. L., Donohue-Rolfe, A., Jackson, M. P., Olsnes, S., Sandvig, K., et al. (1992). Shiga toxin: Biochemistry, genetics, mode of action, and role in pathogenesis. *Curr. Top. Microbiol. Immunol.* 180, 65–94. doi:10.1007/978-3-642-77238-2_4

Oldfield, L. M., Grzesik, P., Voorhies, A. A., Alperovich, N., MacMath, D., Najera, C. D., et al. (2017). Genome-wide engineering of an infectious clone of herpes simplex virus type 1 using synthetic genomics assembly methods. *Proc. Natl. Acad. Sci. U. S. A.* 114, E8885–E8894. doi:10.1073/pnas.1700534114

Oughtred, R., Stark, C., Breitkreutz, B. J., Rust, J., Boucher, L., Chang, C., et al. (2019). The BioGRID interaction database: 2019 update. *Nucleic Acids Res.* 47, D529–D541. doi:10.1093/nar/gky1079

Park, H., Valencia-Gallardo, C., Sharff, A., Tran Van Nhieu, G., and Izard, T. (2011). Novel vinculin binding site of the IpaA invasin of Shigella. *J. Biol. Chem.* 286, 23214–23221. doi:10.1074/jbc.m110.184283

Pearson, W. R. (2013). An introduction to sequence similarity ("homology") searching. *Curr. Protoc. Bioinforma.* 3, 1. doi:10.1002/0471250953.bi0301s42

Pedraza, L. A., Bautista, J., and Uribe-Velez, D. (2018). Seed-born Burkholderia glumae infects rice seedling and maintains bacterial population during vegetative and reproductive growth stage. *Plant Pathol. J.* 34, 393–402. doi:10.5423/ppj.oa.02.2018.0030

Pineda, S. S., Chaumeil, P. A., Kunert, A., Kaas, Q., Thang, M. W. C., Le, L., et al. (2018). ArachnoServer 3.0: An online resource for automated discovery, analysis and annotation of spider toxins. *Bioinformatics* 34, 1074–1076. doi:10.1093/bioinformatics/btx661

Plano, G. V., and Schesser, K. (2013). The *Yersinia pestis* type III secretion system: Expression, assembly and role in the evasion of host defenses. *Immunol. Res.* 57, 237–245. doi:10.1007/s12026-013-8454-3

Poulos, J., and Farnia, A. (2015). *Production of cannabidiolic acid in yeast*. US10093949B2.

Prasanna, A. N., and Mehra, S. (2013). Comparative phylogenomics of pathogenic and non-pathogenic mycobacterium. *PLoS One* 8, e71248. doi:10.1371/journal.pone.0071248

Prusiner, S. B. (1998). Prions. *Proc. Natl. Acad. Sci. U. S. A.* 95, 13363–13383. doi:10.1073/pnas.95.23.13363

Raetz, C. R., and Whitfield, C. (2002). Lipopolysaccharide endotoxins. *Annu. Rev. Biochem.* 71, 635–700. doi:10.1146/annurev.biochem.71.110601.135414

Raimbault, B., Cointet, J. P., and Joly, P. B. (2016). Mapping the emergence of synthetic biology. *PLoS One* 11, e0161522. doi:10.1371/journal.pone.0161522

Rasool, S., Hussain, T., Khan, S. M., Zehra, A., Tahreem, S., and Kakroo, A. M. (2017). Toxins of *Clostridium perfringens* as virulence factors in animal diseases. *J. Pharmacogn. Phytochemistry* 6, 2155–2164.

Raymond, B., Young, J. C., Pallett, M., Endres, R. G., Clements, A., and Frankel, G. (2013). Subversion of trafficking, apoptosis, and innate immunity by type III secretion system effectors. *Trends Microbiol.* 21, 430–441. doi:10.1016/j.tim.2013.06.008

Reactome (2019). Reactome. Available at: https://reactome.org/ (Accessed November 18, 2019).

Reboud, E., Basso, P., Maillard, A. P., Huber, P., and Attree, I. (2017). Exolysin shapes the virulence of *Pseudomonas aeruginosa* clonal outliers. *Toxins (Basel)* 9, 364. doi:10.3390/toxins9110364

RefSeq (2019). Growth statistics. Available at: https://www.ncbi.nlm.nih.gov/refseq/statistics/2019.

Reperant, L. A., and Osterhaus, A. (2017). AIDS, avian flu, SARS, MERS, ebola, Zika. What next? *Vaccine* 35, 4470–4474. doi:10.1016/j.vaccine.2017.04.082

Roly, Z. Y., Hakim, M. A., Zahan, A. S., Hossain, M. M., and Reza, M. A. (2015). ISOB: A database of indigenous snake species of Bangladesh with respective known venom composition. *Bioinformation* 11, 107–114. doi:10.6026/97320630011107

Romani, B., and Engelbrecht, S. (2009). Human immunodeficiency virus type 1 vpr: Functions and molecular interactions. *J. Gen. Virol.* 90, 1795–1805. doi:10.1099/vir.0.011726-0

Rooijakkers, S. H., van Kessel, K. P., and van Strijp, J. A. (2005). Staphylococcal innate immune evasion. *Trends Microbiol.* 13, 596–601. doi:10.1016/j.tim.2005.10.002

Roux, D., Danilchanka, O., Guillard, T., Cattoir, V., Aschard, H., Fu, Y., et al. (2015). Fitness cost of antibiotic susceptibility during bacterial infection. *Sci. Transl. Med.* 7, 297ra114. doi:10.1126/scitranslmed.aab1621

Rudel, T., Scheurerpflug, I., and Meyer, T. F. (1995). Neisseria PilC protein identified as type-4 pilus tip-located adhesin. *Nature* 373, 357–359. doi:10.1038/373357a0

Sarovich, D. S., Price, E. P., Webb, J. R., Ward, L. M., Voutsinos, M. Y., Tuanyok, A., et al. (2014). Variable virulence factors in Burkholderia pseudomallei (melioidosis) associated with human disease. *PLoS One* 9, e91682. doi:10.1371/journal.pone.0091682

Sassetti, C. M., and Rubin, E. J. (2003). Genetic requirements for mycobacterial survival during infection. *Proc. Natl. Acad. Sci. U. S. A.* 100, 12989–12994. doi:10.1073/pnas.2134250100

Sayers, S., Li, L., Ong, E., Deng, S., Fu, G., Lin, Y., et al. (2019). Victors: A web-based knowledge base of virulence factors in human and animal pathogens. *Nucleic Acids Res.* 47, D693–D700. doi:10.1093/nar/gky999

Schmid-Hempel, P. (2009). Immune defence, parasite evasion strategies and their relevance for 'macroscopic phenomena' such as virulence. *Phil. Trans. R. Soc. B* 364, 85–98. doi:10.1098/rstb.2008.0157

Schmitt, C. K., Meysick, K. C., and O'Brien, A. D. (1999). Bacterial toxins: Friends or foes? *Emerg. Infect. Dis.* 5, 224–234. doi:10.3201/eid0502.990206

Schuelke, T. A., Wu, G., Westbrook, A., Woeste, K., Plachetzki, D. C., Broders, K., et al. (2017). Comparative genomics of pathogenic and nonpathogenic beetle-vectored fungi in the genus Geosmithia. *Genome Biol. Evol.* 9, 3312–3327. doi:10.1093/gbe/evx242

Segura, M., Fittipaldi, N., Calzas, C., and Gottschalk, M. (2017). Critical Streptococcus suis virulence factors: Are they all really critical? *Trends Microbiol.* 25, 585–599. doi:10.1016/j.tim.2017.02.005

Serpinskii, O. I., Kochneva, G. V., Urmanov, I., Sivolobova, G. F., and Riabchikova, E. I. (1996). Construction of recombinant variants or orthopoxviruses by inserting foreign genes into intragenic region of viral genome. *Mol. Biol.* 30, 1055–1065.

Shah, P. S., Link, N., Jang, G. M., Sharp, P. P., Zhu, T., Swaney, D. L., et al. (2018). Comparative flavivirus-host protein interaction mapping reveals mechanisms of dengue and Zika virus pathogenesis. *Cell* 175, 1931–1945. doi:10.1016/j.cell.2018.11.028

Shames, S. R., and Finlay, B. B. (2010). Breaking the stereotype: Virulence factor-mediated protection of host cells in bacterial pathogenesis. *PLoS Pathog.* 6, e1001057. doi:10.1371/journal.ppat.1001057

Shames, S. R., Deng, W., Guttman, J. A., de Hoog, C. L., Li, Y., Hardwidge, P. R., et al. (2010). The pathogenic *E. coli* type III effector EspZ interacts with host CD98 and facilitates host cell prosurvival signalling. *Cell. Microbiol.* 12, 1322–1339. doi:10.1111/j.1462-5822.2010.01470.x

Shaver, C. M., and Hauser, A. R. (2004). Relative contributions of *Pseudomonas aeruginosa* ExoU, ExoS, and ExoT to virulence in the lung. *Infect. Immun.* 72, 6969–6977. doi:10.1128/iai.72.12.6969-6977.2004

Shen, Y., Chen, L., Wang, M., Lin, D., Liang, Z., Song, P., et al. (2017). Flagellar hooks and hook protein FlgE participate in host microbe interactions at immunological level. *Sci. Rep.* 7, 1433. doi:10.1038/s41598-017-01619-1

Skurnik, D., Roux, D., Cattoir, V., Danilchanka, O., Lu, X., Yoder-Himes, D. R., et al. (2013). Enhanced *in vivo* fitness of carbapenem-resistant oprD mutants of *Pseudomonas aeruginosa* revealed through high-throughput sequencing. *Proc. Natl. Acad. Sci. U. S. A.* 110, 20747–20752. doi:10.1073/pnas.1221552110

Slusarczyk, A. L., Lin, A., and Weiss, R. (2012). Foundations for the design and implementation of synthetic genetic circuits. *Nat. Rev. Genet.* 13, 406–420. doi:10.1038/nrg3227

Smatti, M. K., Cyprian, F. S., Nasrallah, G. K., Al Thani, A. A., Almishal, R. O., and Yassine, H. M. (2019). Viruses and autoimmunity: A review on the potential interaction and molecular mechanisms. *Viruses* 11, 762. doi:10.3390/v11080762

Smedley, J. G., 3rd, Fisher, D. J., Sayeed, S., Chakrabarti, G., and McClane, B. A. (2004). The enteric toxins of *Clostridium perfringens*. *Rev. Physiol. Biochem. Pharmacol.* 152, 183–204. doi:10.1007/s10254-004-0036-2

Smith, H. O., Hutchison, C. A., 3rd, Pfannkoch, C., and Venter, J. C. (2003). Generating a synthetic genome by whole genome assembly: φX174 bacteriophage from synthetic oligonucleotides. *Proc. Natl. Acad. Sci. U. S. A.* 100, 15440–15445. doi:10.1073/pnas.2237126100

Stapleton, P. D., and Taylor, P. W. (2002). Methicillin resistance in *Staphylococcus aureus*: Mechanisms and modulation. *Sci. Prog.* 85, 57–72. doi:10.3184/003685002783238870

Stebbins, C. E., and Galan, J. E. (2001). Structural mimicry in bacterial virulence. *Nature* 412, 701–705. doi:10.1038/35089000

Straus, M. R., and Whittaker, G. R. (2017). A peptide-based approach to evaluate the adaptability of influenza A virus to humans based on its hemagglutinin proteolytic cleavage site. *PLoS One* 12, e0174827. doi:10.1371/journal.pone.0174827

Suzek, B. E., Wang, Y., Huang, H., McGarvey, P. B., Wu, C. H., and UniProt, C. (2015). UniRef clusters: A comprehensive and scalable alternative for improving sequence similarity searches. *Bioinformatics* 31, 926–932. doi:10.1093/bioinformatics/btu739

Sweet, C. R., Conlon, J., Golenbock, D. T., Goguen, J., and Silverman, N. (2007). YopJ targets TRAF proteins to inhibit TLR-mediated NF-kappaB, MAPK and IRF3 signal transduction. *Cell. Microbiol.* 9, 2700–2715. doi:10.1111/j.1462-5822.2007.00990.x

Sweigard, J. A., Chumley, F. G., and Valent, B. (1992). Cloning and analysis of CUT1, a cutinase gene from Magnaporthe grisea. *Molec. Gen. Genet.* 232, 174–182. doi:10.1007/bf00279994

Tehel, A., Vu, Q., Bigot, D., Gogol-Doring, A., Koch, P., Jenkins, C., et al. (2019). The two prevalent genotypes of an emerging infectious disease, deformed wing virus, cause equally low pupal mortality and equally high wing deformities in host honey bees. *Viruses* 11, 114. doi:10.3390/v11020114

Telling, G. C., Scott, M., Mastrianni, J., Gabizon, R., Torchia, M., Cohen, F. E., et al. (1995). Prion propagation in mice expressing human and chimeric PrP transgenes implicates the interaction of cellular PrP with another protein. *Cell* 83, 79–90. doi:10.1016/0092-8674(95)90236-8

The, C. (2019). Gene ontology, the gene ontology resource: 20 years and still GOing strong. *Nucleic Acids Res.* 47, D330–D338.

Tillett, D., Dittmann, E., Erhard, M., von Dohren, H., Borner, T., and Neilan, B. A. (2000). Structural organization of microcystin biosynthesis in microcystis aeruginosa PCC7806: An integrated peptide-polyketide synthetase system. *Chem. Biol.* 7, 753–764. doi:10.1016/s1074-5521(00)00021-1

Tsang, T. M., Felek, S., and Krukonis, E. S. (2010). Ail binding to fibronectin facilitates *Yersinia pestis* binding to host cells and Yop delivery. *Infect. Immun.* 78, 3358–3368. doi:10.1128/iai.00238-10

U.S. Department of Health and Human Services (2020). *Biosafety in microbiological and biomedical laboratories*. Sixth Edition. Washington DC. https://www.cdc.gov/labs/pdf/CDC-BiosafetyMicrobiologicalBiomedicalLaboratories-2020-P.pdf.

U.S. Department of Justice. Drug Enforcement Agency. Diversion Control Division (2019) Title 21 United States code (USC) controlled substances act. Available at: https://www.deadiversion.usdoj.gov/21cfr/21usc/index.html (Accessed Nover 18, 2019).

UniProt. (2019) UniRef. Available at: https://www.uniprot.org/help/uniref (Accessed November 18, 2019).

UniProt, C. (2019). UniProt: A worldwide hub of protein knowledge. *Nucleic Acids Res.* 47, D506–D515. doi:10.1093/nar/gky1049

UniProt (2019). Animal toxin annotation project. Available at: https://www.uniprot.org/program/Toxins.

United States Department of Agriculture Economic Research Service (2022) Farming and farm income. Available at: https://www.ers.usda.gov/data-products/ag-and-food-statistics-charting-the-essentials/farming-and-farm-income/(Accessed 8 2022).

United States Drug Enforcement Administration (2019). Drug scheduling. Available at: https://www.dea.gov/drug-scheduling (Accessed October 10, 2019).

Urban, M., Cuzick, A., Rutherford, K., Irvine, A., Pedro, H., Pant, R., et al. (2017). PHI-Base: A new interface and further additions for the multi-species pathogen-host interactions database. *Nucleic Acids Res.* 45, D604–D610. doi:10.1093/nar/gkw1089

US Department of Health and Human Services (2017). Framework for guiding funding decisions about proposed research involving enhanced potential pandemic pathogens. Available at: https://www.phe.gov/s3/dualuse/Documents/p3co.pdf.

US Department of Health and Human Services (2022). Screening framework guidance for providers of synthetic double-stranded DNA. Available at: https://www.phe.gov/Preparedness/legal/guidance/syndna/Pages/default.aspx.

Usmani, S. S., Bedi, G., Samuel, J. S., Singh, S., Kalra, S., Kumar, P., et al. (2017). THPdb: Database of FDA-approved peptide and protein therapeutics. *PLoS One* 12, e0181748. doi:10.1371/journal.pone.0181748

Uzzau, S., and Fasano, A. (2000). Cross-talk between enteric pathogens and the intestine. *Cell. Microbiol.* 2, 83–89. doi:10.1046/j.1462-5822.2000.00041.x

van Der Most, R. G., Murali-Krishna, K., Ahmed, R., and Strauss, J. H. (2000). Chimeric yellow fever/dengue virus as a candidate dengue vaccine: Quantitation of the dengue virus-specific CD8 T-cell response. *J. Virol.* 74, 8094–8101. doi:10.1128/jvi.74.17.8094-8101.2000

Velmurugan, K., Chen, B., Miller, J. L., Azogue, S., Gurses, S., Hsu, T., et al. (2007). *Mycobacterium tuberculosis* nuoG is a virulence gene that inhibits apoptosis of infected host cells. *PLoS Pathog.* 3, e110. doi:10.1371/journal.ppat.0030110

Vickers, C., and Small, I. (2018) The synthetic biology revolution is now – here's what that means. Available at: https://phys.org/news/2018-09-synthetic-biology-revolution.html2018.

Vieira, A., Silva, D. N., Varzea, V., Paulo, O. S., and Batista, D. (2019). Genome-wide signatures of selection in Colletotrichum kahawae reveal candidate genes potentially involved in pathogenicity and aggressiveness. *Front. Microbiol.* 10, 1374. doi:10.3389/fmicb.2019.01374

Vila, J., Marti, S., and Sanchez-Cespedes, J. (2007). Porins, efflux pumps and multidrug resistance in Acinetobacter baumannii. *J. Antimicrob. Chemother.* 59, 1210–1215. doi:10.1093/jac/dkl509

ViralZone (2019). Viralzone news. Available at: https://viralzone.expasy.org/ (Accessed November 18, 2019).

Visiello, R., Colombo, S., and Carretto, E. (2016). *Chapter 3 - Bacillus cereus hemolysins and other virulence factors, the diverse faces of Bacillus cereus*. Amsterdam, Netherlands: Elsevier, 35–44.

von Mering, C., Jensen, L. J., Snel, B., Hooper, S. D., Krupp, M., Foglierini, M., et al. (2005). String: Known and predicted protein-protein associations, integrated and transferred across organisms. *Nucleic Acids Res.* 33, D433–D437. doi:10.1093/nar/gki005

Wang, J., Yin, T., Xiao, X., He, D., Xue, Z., Jiang, X., et al. (2018). StraPep: A structure database of bioactive peptides. *Database (Oxford)* 2018, bay038. doi:10.1093/database/bay038

Wattam, A. R., Davis, J. J., Assaf, R., Boisvert, S., Brettin, T., Bun, C., et al. (2017). Improvements to PATRIC, the all-bacterial bioinformatics database and analysis resource center. *Nucleic Acids Res.* 45, D535–D542. doi:10.1093/nar/gkw1017

Welch, R. A., Burland, V., Plunkett, G., 3rd, Redford, P., Roesch, P., Rasko, D., et al. (2002). Extensive mosaic structure revealed by the complete genome sequence of uropathogenic *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A.* 99, 17020–17024. doi:10.1073/pnas.252529799

Wells, G. A., Scott, A. C., Johnson, C. T., Gunning, R. F., Hancock, R. D., Jeffrey, M., et al. (1987). A novel progressive spongiform encephalopathy in cattle. *Vet. Rec.* 121, 419–420. doi:10.1136/vr.121.18.419

Whitworth, T., Popov, V. L., Yu, X. J., Walker, D. H., and Bouyer, D. H. (2005). Expression of the *Rickettsia prowazekii* pld or tlyC gene in *Salmonella enterica* serovar Typhimurium mediates phagosomal escape. *Infect. Immun.* 73, 6668–6673. doi:10.1128/iai.73.10.6668-6673.2005

Wilesmith, J. W. (1994). Bovine spongiform encephalopathy and related diseases: An epidemiological overview. *N. Z. Vet. J.* 42, 1–8. doi:10.1080/00480169.1994.35774

Will, R. G., Ironside, J. W., Zeidler, M., Cousens, S. N., Estibeiro, K., Alperovitch, A., et al. (1996). A new variant of Creutzfeldt-Jakob disease in the UK. *Lancet* 347, 921–925. doi:10.1016/s0140-6736(96)91412-9

Winsor, G. L., Griffiths, E. J., Lo, R., Dhillon, B. K., Shay, J. A., and Brinkman, F. S. (2016). Enhanced annotations and features for comparing thousands of Pseudomonas genomes in the Pseudomonas genome database. *Nucleic Acids Res.* 44, D646–D653. doi:10.1093/nar/gkv1227

Wishart, D., Arndt, D., Pon, A., Sajed, T., Guo, A. C., Djoumbou, Y., et al. (2015). T3DB: The toxic exposome database. *Nucleic Acids Res.* 43, D928–D934. doi:10.1093/nar/gku1004

Wong, G., Kobinger, G. P., and Qiu, X. (2014). Characterization of host immune responses in Ebola virus infections. *Expert Rev. Clin. Immunol.* 10, 781–790. doi:10.1586/1744666x.2014.908705

Xiong, Z., Jiang, Y., Qi, D., Lu, H., Yang, F., Yang, J., et al. (2009). Complete genome sequence of the extremophilic Bacillus cereus strain Q1 with industrial applications. *J. Bacteriol.* 191, 1120–1121. doi:10.1128/jb.01629-08

Xu, S. X., and McCormick, J. K. (2012). Staphylococcal superantigens in colonization and disease. *Front. Cell. Infect. Microbiol.* 2, 52. doi:10.3389/fcimb.2012.00052

Xu, X., Liu, W., Tian, S., Wang, W., Qi, Q., Jiang, P., et al. (2018). Petroleum hydrocarbon-degrading bacteria for the remediation of oil pollution under aerobic conditions: A perspective analysis. *Front. Microbiol.* 9, 2885. doi:10.3389/fmicb.2018.02885

Yongkiettrakul, S., Maneerat, K., Arechanajan, B., Malila, Y., Srimanote, P., Gottschalk, M., et al. (2019). Antimicrobial susceptibility of Streptococcus suis isolated from diseased pigs, asymptomatic pigs, and human patients in Thailand. *BMC Vet. Res.* 15, 5. doi:10.1186/s12917-018-1732-5

Yoon, S. H., Park, Y. K., and Kim, J. F. (2015). PAIDB v2.0: Exploration and analysis of pathogenicity and resistance islands. *Nucleic Acids Res.* 43, D624–D630. doi:10.1093/nar/gku985

Zakham, F., Aouane, O., Ussery, D., Benjouad, A., and Ennaji, M. M. (2012). Computational genomics-proteomics and Phylogeny analysis of twenty one mycobacterial genomes (Tuberculosis & non Tuberculosis strains). *Microb. Inf. Exp.* 2, 7. doi:10.1186/2042-5783-2-7

Zaluga, J., Stragier, P., Baeyen, S., Haegeman, A., Van Vaerenbergh, J., Maes, M., et al. (2014). Comparative genome analysis of pathogenic and non-pathogenic Clavibacter strains reveals adaptations to their lifestyle. *BMC Genomics* 15, 392. doi:10.1186/1471-2164-15-392

Zamyatnin, A. A., Borchikov, A. S., Vladimirov, M. G., and Voronina, O. L. (2006). The EROP-Moscow oligopeptide database. *Nucleic Acids Res.* 34, D261–D266. doi:10.1093/nar/gkj008

Zhang, Y., Aevermann, B. D., Anderson, T. K., Burke, D. F., Dauphin, G., Gu, Z., et al. (2017). Influenza Research Database: An integrated bioinformatics resource for influenza virus research. *Nucleic Acids Res.* 45, D466–D474. doi:10.1093/nar/gkw857

Zhang, H. (2003). Lethality in mice infected with recombinant vaccinia virus expressing hepatitis C virus core protein. *Hepatobiliary Pancreat. Dis. Int.* 2, 374–382.

Zhou, C. E., Smith, J., Lam, M., Zemla, A., Dyer, M. D., and Slezak, T. (2007). MvirDB--a microbial database of protein toxins, virulence factors and antibiotic resistance genes for bio-defence applications. *Nucleic Acids Res.* 35, D391–D394. doi:10.1093/nar/gkl791

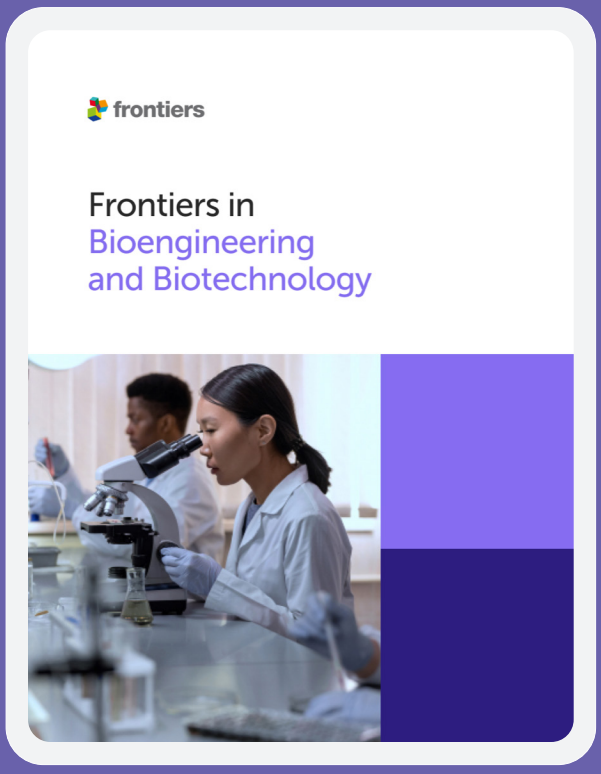
Frontiers in
Bioengineering and Biotechnology

Accelerates the development of therapies, devices, and technologies to improve our lives

A multidisciplinary journal that accelerates the development of biological therapies, devices, processes and technologies to improve our lives by bridging the gap between discoveries and their application.

Discover the latest Research Topics

See more →



Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact