BIOSAFETY OF GENETICALLY MODIFIED ORGANISMS 3

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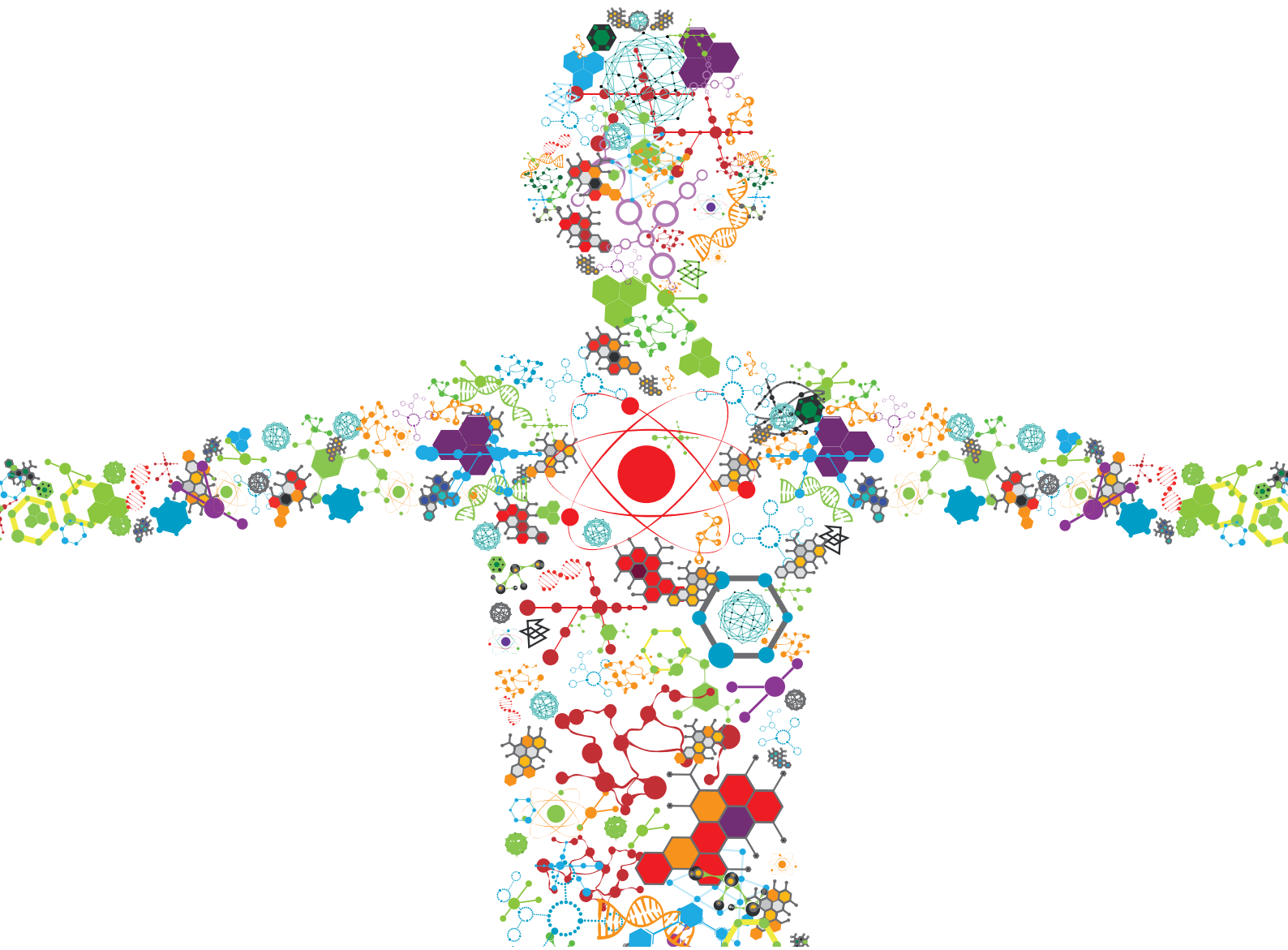

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BIOSAFETY OF GENETICALLY MODIFIED ORGANISMS 3

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EDITORIAL
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Editorial: Biosafety of Genetically Modified Organisms 3. A Collection of Publications from the 15th International Society for Biosafety Research Symposium

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Keywords: biosafety, regulation, risk assessment, sustainable biotechnology, policy innovation

Editorial on the Research Topic

Editorial: Biosafety of Genetically Modified Organisms 3. A Collection of Publications from the 15th International Society for Biosafety Research Symposium

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The ISBR Symposium [previously known as the International Symposium on Biosafety of Genetically Modified Organisms (ISBGMO)] is an international meeting organized by the International Society for Biosafety Research (ISBR), a society whose membership is composed of individuals with expertise and interest in regulations, risk assessments, and research associated with the sustainable use of biotechnology (http://www.isbr.info/). These symposia have been offered biennially since 1990, at various locations throughout the world, as a unique opportunity for public and private sector research scientists, regulators, technology developers, nongovernment organizations and others to share their experience and expertise and to discuss biosafety related to the application of biotechnology. As with past symposia, ISBR hosted a research topic titled "Biosafety of Genetically Modified Organisms 3" in Frontiers in Bioengineering and Biotechnology: section Biosafety and Biosecurity, open to the presenters at the most recent 15th ISBR Symposium held in April of 2019 in Tarragona, Spain (**Figure 1**).

The goals of the ISBR Symposium are 1) to share biosafety research and application and chart new research directions, 2) to foster productive dialogue and multidisciplinary approaches, and 3) to embrace perspectives from all parts of the globe. The emphasis by ISBR for these symposia has evolved over the years. Early symposia were focused mainly on presentations of the results of research related to risks of biotechnology and environmental risk assessment. More recent meetings have increasingly included presentations, workshops, and forward-thinking discussions on the regulation of biotechnology, data requirements, and the relevance of risk assessment research for decision-making, policy development and encouraging innovation. This shifting emphasis highlights the distinctiveness of this symposium compared to other more academic scientific meetings that may feature biosafety among their sessions. ISBR also has intentionally broadened the scope of the symposia to include new and emerging applications of biotechnology with implications for regulatory research and policy that are different than the "traditional" genetically modified organisms of the past.

FIGURE 1 | The 15th ISBR Symposium was held in April 2019 in Tarragona, Spain.

The 15th ISBR Symposium, with 274 attendees participating from 42 countries, had as its theme "New Horizons in Biotechnology: Risk Analysis for a Sustainable Future". Around this central theme, the program included a series of presentations in four topical plenary sessions: 1) Communications and Engagement with Policy and Public Audiences; 2) Environmental Risk Assessment and Regulation of Gene Edited Products; 3) Food Safety Assessment of Novel Molecules–What Does the Future Hold; and 4) Challenges in the Development and Adoption of Novel Biotechnologies. In addition to these plenary sessions, the symposium included over 20 organized sessions and workshops offered in parallel on a range of topics, numerous Pecha Kucha and traditional poster presentations, and accommodated some smaller satellite conferences in its margins. Out of the diverse presentations, the society has assembled a collection of 17 peer-reviewed publications representative of the different kinds of thought-provoking topics presented and discussed at the ISBR symposium.

The research topic from the 15th ISBR Symposium includes four timely and compelling "policy and practice reviews," perhaps most representative of the novelty of the ISBR Symposium. Two of these articles deal specifically with the most current new breeding techniques broadly described as "gene-editing" that have garnered a great deal of attention and discussion among the biotechnology research and regulatory communities. In fact, one of the plenary session topics at the symposium was devoted to environmental risk assessment and regulation of gene-edited products. One of the reviews is based on a presentation in that plenary session and presents an important discussion of the regulatory experience in Argentina with gene-edited products compared to "traditional" GMOs (genetically modified organisms), and the potential for changes in regulatory practices to encourage innovation (Whelan et al.). The second review describes Japan's progress in developing and implementing a regulatory approach for genome-edited organisms within the existing biosafety framework (Tsuda et al.). Governments worldwide are similarly working to define their approach to regulating gene-edited organisms, recognizing the great potential for applications of this technology in human health and agriculture.

Another important policy and practice review discusses regulation of GMOs being developed for invasive species control, specifically using gene drive applications (Mitchell and Bartsch). Gene drives are another hot regulatory topic because of the increased potential for transboundary movement and replacement of target populations associated with this technology. This article identifies information gaps and considers scenarios for safely releasing gene drive organisms into the environment. The fourth policy and practice review takes a close look at biosafety and biosecurity of GMOs in containment and provides a global overview of how regulatory frameworks have evolved to manage these (Beeckman and Rüdelsheim). This article includes a very useful discussion of different ways biosafety and biosecurity can be defined and the scope of biosafety as it overlaps with biosecurity.

The research topic also includes three progressive general "review" articles. Teem et al. describe different approaches for genetic biocontrol, including gene drives, and the regulatory considerations of each to minimize potential harm to the environment. Another review discusses the deliberations taking place on "synthetic biology" under the Convention on Biological Diversity (CBD), a multilateral treaty that has significant implications for regulation of biotechnology (Keiper and Atanassova). This review describes synthetic biology as "part of the continuum of modern biotechnological development"; as such, the adequacy of existing regulatory mechanisms for "living modified organisms" to also regulate the "new" biotechnologies encompassed by "synthetic biology" has become central to the CBD deliberations. A third article reviews ongoing discussions about the appropriate tests and use of endpoints needed to inform non-target arthropod assessment of crop plants with pesticidal properties, especially for new technologies that have a different mode of action than the more familiar Bt Cry proteins such as traits based on RNA interference (Roberts et al.).

The 15th ISBR Symposium research topic includes four 'original research' articles. Two of these address some of the most common environmental and food safety concerns associated with genetically engineered crops. Xu et al. present

the results of a study to understand the potential risk to nontarget organisms due to changes in herbivore-induced plant volatiles of an insect resistant Bt maize compared to non-Bt maize. This study concluded that the changes in plant volatiles do not affect the behavior of *Trichogramma* egg parasitoids, considered beneficial in controlling lepidopteran pests of maize. Bressan et al. studied the potential occurrence of gene flow from sugarcane cultivars to wild relatives and the nutritional composition of sugarcane cultivars in Brazil, to use as baseline studies for risk assessment of genetically engineered sugarcane.

Another article, describing original research that has received marked attention, reports on the first limited field release of a genetically engineered, "self-limiting" agricultural pest insect, the diamondback moth which is a serious global pest of crucifers, and the series of studies that were conducted to evaluate its potential as a biologically-based approach to crop pest management (Shelton et al.). In addition, an article (by the same first author) presents the results of research demonstrating the impact in the market value chain in Bangladesh of a genetically engineered insect resistant brinjal (eggplant), one of the first genetically engineered food crops approved for cultivation in a developing country (Shelton et al.). This article was complemented by a "brief research report" considering the biosafety management measures as well as socio-economic impacts and challenges of this same insect resistant brinjal in Bangladesh (Haque and Saha).

The remaining articles in the 15th ISBR Symposium research topic are five "perspective" pieces. Perspectives are welcomed additions to the research topic, as these short articles offer an opportunity for authors to capture their thoughts and experiences on specific topics as presented at the symposium. Four of these perspectives come from Latin America and each of these shares lessons that should be applicable to regulatory systems across the globe. One of these discusses how different countries in the Americas have applied the concept of familiarity in risk assessments of transgenic crops and effectively demonstrates how this concept has become a key element of the risk assessment process (Capalbo et al.). Another paper describes the establishment in the regulatory system of Paraguay of a simplified procedure for evaluating the safety of GM crops that allows the use of risk assessments and decision documents already issued in another country for the same GM event (Candia et al.). A similar idea, the transportability of conclusions from confined field trials from Brazil to Argentina, is discussed in a perspective that uses the virus resistant GM bean developed in Brazil as a case study (Vesprini et al.). Another perspective is shared in an article from Argentina that discusses the challenge for locally developed GM crops to reach the market compared to those coming from private industry, and the need for a regulatory affairs platform for the public research system (Lewi and Vicién). One more perspective describes the experience of developing an effective insect resistance management (IRM) strategy for Bt maize following the discovery of resistance development in the target insect pest in South Africa, with implications for developing more effective IRM strategies for other insect resistant maize in Africa (Bouwer).

ISBR gratefully acknowledges the contribution from all the authors to this research topic. The society has identified an important niche to fill in the scientific community, and the diversity of topics and article types published as part of this research topic exemplify the goals and impact of the ISBR Symposium. The society intends to continue to bring together this unique group to share perspectives, learn from experiences and plan for sound scientific global approaches to biosafety in the future. The 16th ISBR Symposium will take place in April 2022 in St. Louis, Missouri United States.

AUTHOR CONTRIBUTIONS

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

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ORIGINAL RESEARCH
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Effects of *Bacillus thuringiensis* Genetic Engineering on Induced Volatile Organic Compounds Emission in Maize and the Attractiveness to a Parasitic Wasp

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In order to control lepidopteran and coleopteran insects, the genes expressing *Bacillus thuringiensis* (*Bt*) insecticidal proteins have been transferred into crops. Ecological risk assessments of the transgenic plants have included impacts on non-target entomophagous insects, such as parasitoid wasps. Herbivore-induced plant volatiles are considered to be important defensive traits of plants because these compounds play as an important role in recruitment of natural enemies. Here, we evaluated induced volatile emissions of maize seedlings of two *Bt* cultivars (5422Bt1, event Bt11 and 5422CBCL, event Mon810), and their nearly isogenic non-*Bt* line 5422. We damaged plants mechanically and then applied with the regurgitant of *Spodoptera litura* (F.) caterpillars (Lepidoptera: Noctuidae), or treated the plants with the plant hormone jasmonic acid (JA), to trigger similar defensive responses of plants. Compared to the non-*Bt* isoline 5422 and the *Bt* maize 5422CBCL, the other *Bt* maize 5422Bt1 released more (3*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) when they were all treated by artificial wounds and caterpillar regurgitant; and released more linalool, DMNT and (*E*)-β-farnesene when applied with JA solution. As a result, the total volatile emission of the 5422Bt1 was highest. However, the difference in volatile emission did not affect the attractiveness of the *Bt* maize plants to the egg parasitoid *Trichogramma ostriniae* Pang et Chen (Hymenoptera: Trichogrammatidae) compared to the nearly isogenic non-*Bt* plants. The variability of induced volatiles of maize cultivars derived from conventional breeding programs and transgenic methods are discussed.

Keywords: tritrophic interactions, leaf-chewing insects, genetically modified organism, plant-insect interactions, egg parasitoids

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INTRODUCTION

In the last two decades, farmers around the world have rapidly adopted genetically modified (GM) crops with biotech-derived beneficial traits, e.g., herbicide tolerance and pest resistance (James, 2017). Those traits have benefited humans by increasing crop productivities and reducing environmental pollutions that can be caused by applications of chemical pesticides (Cattaneo et al., 2006; Romeis et al., 2006; Wu et al., 2008; James, 2017).

Bt crops are continuously expressing insecticidal proteins (*δ*-endotoxin), which are derived from soil bacterium *Bacillus thuringiensis* (*Bt*) Berliner. Those crops are designed to defend themselves against herbivores of Coleoptera and Lepidoptera. Expressing *Bt* proteins may change some defensive characteristics of plants to some non-target organisms. For example, several *Bt* maize cultivars and a *Bt* cotton line were found to be more susceptible to aphid damages than their respective non-*Bt* isolines in the laboratory and/or in the field (Faria et al., 2007; Hagenbucher et al., 2013).

For some entomophagous arthropods (predators and parasitoids) that feed/host on non-target insects (e.g., aphids), their population has not decreased significantly in a *Bt* crop field compared to a conventional crop field (Dutton et al., 2002, 2003; Lumbierres et al., 2011; Yao et al., 2016; Romeis et al., 2019). The field studies seem to support that *Bt* proteins are not likely to be toxic to entomophagous arthropods in the natural environment. Indeed, similar conclusions are also drawn from laboratory studies: *Bt* proteins in crops have not been reported to harm entomophagous insects when their prey/hosts are not susceptible to *Bt* toxins (i.e., *Bt*-resistant herbivores) or sap-sucking insects such as aphids that feed on plant phloem sap where *Bt* toxins are with trace amounts. For example, several parasitoid species that hosted on *Bt*-resistant *Plutella xylostella* (L.) caterpillars developed with negligible negative effects (Schuler et al., 1999, 2004; Chen et al., 2008; Liu et al., 2011). The phenomenon was further confirmed by physiological data: larvae of the endoparasitoid species *Diadegma insulare* (Cresson) were found to be exposed to a biologically active form of *Bt* proteins in the *Bt*-resistant hosts, but the survival of the parasitoids did not significantly decrease (Chen et al., 2008). In addition, with the damage by the *Bt*-resistant *P. xylostella,* the attractiveness to a few parasitoid species of *Bt* rape plants was as strong as that obtained from conventional hybrids (Schuler et al., 1999, 2003; Liu et al., 2011). When feeding on *Bt* cotton plants, bodies of the sap-sucking herbivore *Ferrisia virgata* Cockerell did not contain detectable amount of *Bt* proteins, and the survival and development of the herbivore species and its predator *Cryptolaemus montrouzieri* Mulsant were negligibly affected by the *Bt* crop (Wu et al., 2014). Therefore, *Bt* proteins in crops seem not to be poisonous to parasitoids and predators, which indicates releasing natural enemies is still likely to be an effective way to control non-target or *Bt*-resistant pests in *Bt* fields (Romeis et al., 2019).

In maize, transformations of foreign genes may quantitatively change the emission of the herbivore-induced plant volatiles (HIPVs), which undertake several ecological functions including attracting natural enemies of herbivores (Heil, 2014). The *Bt* maize plant (N4640Bt, event Bt11) emitted a few volatile compounds in a smaller amount than did its isogenic non-*Bt* line when they were both treated by mechanical injury and then applied with herbivore regurgitant, possibly because the *Bt* maize allocated some resources on the biosynthesis of *Bt* proteins, and as a result, less resources to produce HIPVs (Turlings et al., 2005). The differences did not affect their attractiveness to two endoparasitoid species (Turlings et al., 2005). *Bt* transgenic events do not necessarily result in a shift of HIPVs in maize. For example, the *Bt* maize plant (DKC61-25, event Mon810) emitted similar amounts of HIPVs with its isogenic non-*Bt* line when they were damaged by the same controlled method, artificial wounds and caterpillar regurgitant (Dean and De Moraes, 2006). Since GM maize plants with *Bt* genes have been adopted on a large-scale worldwide, the effects of different transgenic events on emissions of induced volatile organic compounds (VOCs) and tritrophic interactions with different insect species need long-term evaluations.

The tobacco cutworm *S. litura* is widespread throughout tropical and subtropical Asia. Larvae of this insect feed on a wide spectrum of agricultural and horticultural crops and have caused severe damage (Wei et al., 2004). Chemical pesticides are not sufficient to control this species on some crops because larval resistance develops quickly, and as a result, biological controls with parasitoid wasps possibly act as an important alternative (Kuhar et al., 2004; Wei et al., 2004). The egg parasitoid *T. ostriniae*, endemic to China, is an important candidate to control several lepidopteran pests, such as the European corn borer (Hoffmann et al., 1995; Kuhar et al., 2004; Gardner et al., 2007). Moth eggs of many species in the Noctuidae, Pyralidae, and Plutellidae of the Lepidoptera have experienced high levels of parasitism by the parasitoids (Hoffmann et al., 1995). *S. litura* is one of the host species of the parasitic wasps (Kuhar et al., 2004). Furthermore, caterpillar-damaged plants are commonly reported to attract egg parasitoids (Reddy et al., 2002; Peñaflor et al., 2011b; War et al., 2016; Michereff et al., 2019; Ortiz-Carreon et al., 2019). However, our knowledge on how *Bt* transgenic events affect the host-finding behaviors of egg parasitoids is relatively limited. In this study, we compared the induced VOCs of two transgenic *Bt* maize plants (5422Bt1, event Bt11 and 5422CBCL, event Mon810), and their nearly isogenic non-*Bt* cultivar 5422 when they were treated by artificial wounds and caterpillar regurgitant, or the plant hormone jasmonic acid (JA). In addition, the attractiveness of intact and induced plants of the three cultivars to the generalist egg parasitoid wasps was also tested and compared.

MATERIALS AND METHODS

Plants and Insects

Seeds of the two transgenic maize plants 5422Bt1 (event Bt11) and 5422CBCL (event Mon810) expressing Cry1Ab and the nearly isogenic non-*Bt* cultivar (5422) of the seed company Beck's Hybrids, Atlanta, Indiana, USA were provided by Dr. Cindy Nakatsu in the Agronomy Department of Purdue University, USA. All plants were cultivated in greenhouse (25°C, L:D = 16:8 h). Larvae of the generalist herbivore *S. litura* were reared on an artificial diet in the laboratory (Qi et al., 2000). The generalist egg parasitoid *T. ostriniae* (Hymenoptera: Trichogrammatidae) was originally provided by Guangdong Entomological Institute, Guangzhou, China. The parasitoids were reared on the eggs of *Pyrausta nubilalis* Hübner (Lepidoptera: Pyralidae), which were bought from an insect-rearing company. Some *S. litura* caterpillars (3rd instar) were reared in a plastic box (3 × 10 × 10 cm) and fed with 5422

maize leaves for 24 h, and then their regurgitant was collected by a pipette twice a day (20 μL, Eppendorf) (Turlings et al., 1993). Maize seedlings (14-day old) were treated with different methods to collect VOCs and for bioassays of the parasitoid species: plants damaged by scissors (1 cm length × 15 times on 2nd and 3rd leaves, i.e., two biggest leaves of maize seedlings) and then applied with 10 μL caterpillar regurgitant; or alternatively, the 2nd and 3rd leaves were painted with 20 μL plant hormone JA solution (4 mM), respectively. We tested plants immediately after the induction by caterpillar regurgitant, because they started to release plant volatiles shortly after the treatment (Erb et al., 2015). The plants treated by JA solution were left for about 14 h (overnight, L:D = 16:8 h) before used for the same tests, with consideration of that JA responses are likely to show an apparent increase in a few hours after an exogenous application of JA solution (Bruinsma et al., 2009).

Olfactory Preference of Parasitoids

The preference of the parasitoid *T. ostriniae* were tested with a Y-tube olfactometer (ID = 2 cm, arm length = 16 cm). Each arm of the olfactometer was connected by a Teflon tube to an empty glass container (about 1 L) or a glass container of the same type where a treated or an intact plant was placed. A cleaned and humidified constant airflow (0.7 L/min) passed through the odor source and then entered in the olfactometer in which a naive parasitoid wasp was released. The parasitoid was considered to have made a choice when it chose one arm, walked to the odor source for more than 3 cm and stayed in that region for more than 30 s. When the wasp had made a choice or the testing time was up to 10 min (recorded as non-choice), the wasp was removed from the system, and then another naive wasp was released. To eliminate biased choices toward one arm position in each replicate, eight wasps were tested first (released one by one), and then the position of olfactometer was reversed. Then, another eight wasps were tested with the same olfactometer (released one by one). Each experiment was replicated four times (64 wasps in total).

Headspace Volatile Sampling and Analyses

To identify and quantify the VOCs emitted by *Bt*/non-*Bt* maize plants under different treatments (caterpillar regurgitant and JA), each plant (14-day old) was put into a glass container (about 1 L) at the room temperature (25°C). VOCs were collected with Tenax filter (50 mg, 60–80 mesh, Supleco, Bellefonte, PA, USA) and the headspace air was pumped through the filter at a speed of 0.7 L/min for 4 h. A cleaned and humidified constant airflow entered the system with the same speed. After each collection, VOCs were eluted from the filters with 200 μL hexane (Sigma) five times. Then the elution was concentrated to about 500 μL with a gentle stream of nitrogen. The samples were then stored at −20°C until chemical analyses. Each experiment was replicated four times. In order to quantify VOCs, 10 μL of the internal standard, n-octane (200 ng in 10 μL hexane) was added to each sample. VOCs were analyzed with an Agilent 6890 gas chromatograph, connected with Agilent 5973 Network mass selective detector. A 2 μL aliquot of each sample was injected with splitless mode (280 °C) onto a non-polar column (HP-5 ms, 30 m, 0.25 mm ID, 0.25 μm film thickness, Agilent J&W Scientific, USA) at an initial column temperature of 50°C for 3 min, and then temperature was increased at a rate of 8°C per minute to 230 °C, and then the column temperature was held for 7.5 min. Helium at constant flow (0.9 ml/min) was used as carrier gas. Identifications of the compounds were initially carried out by mass spectrometry analysis: i.e., compounds were identified by comparing the mass spectra obtained from the samples with those from a reference database (NIST mass spectral library). Then those compounds were confirmed with authentic ones bought from Sigma-Aldrich (USA).

Statistics

For the olfactometer data, statistical analyses were performed with SigmaPlot 14.0 (Systat Software Inc., San Jose, CA, USA) with a two-tailed *t*-test. For the quantity of VOCs, a one-way ANOVA was applied by the same software, and a Holm-Sidak *post hoc* analysis was used for pairwise comparisons. Statistical differences ($p < 0.05$) were indicated with different letters in the bar figures, and the detail statistical results were presented in **Supplementary File 1**.

RESULTS

For both *Bt* maize and non-*Bt* maize cultivars, their intact plants were not significantly more attractive to the parasitoid *T. ostriniae* than blank controls (**Table 1**). When treated by caterpillar regurgitant, both *Bt* maize and non-*Bt* maize plants were more attractive to the parasitoids than the blank arm and, by extension, their respective intact plants (**Table 1**). The strong attractiveness was still present when those plants were induced by plant hormone JA (**Table 1**).

The *Bt* maize plants (5422Bt1 and 5422CBCL) were as attractive as their nearly isogenic non-*Bt* line (5422) to the parasitoid *T. ostriniae*, when they were all undamaged, applied with caterpillar regurgitant, or treated by JA (**Table 1**). The attractiveness to the parasitoid species of the 5422Bt1 was not significantly different from 5422CBCL once they were treated in the same way (**Table 1**).

With the treatments of caterpillar regurgitant and JA, all three cultivars released 11 main volatile compounds: (*Z*)-3-hexen-1-yl acetate, (*E*)-β-ocimene, linalool, (3*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), phenethyl acetate, indole, methyl anthranilate, geranyl acetate, (*E*)-β-caryophyllene, (*E*)-α-bergamotene, (*E*)-β-farnesene, and (*E*)-nerolidol (**Figure 1A**). When induced by caterpillar regurgitant, the 5422Bt1 released more DMNT than did 5422CBCL and the non-*Bt* line 5422, which probably resulted in the total volatile emission of 5422Bt1 was also highest (**Figure 1B**). Comparable results occurred when plants were induced by JA: 5422Bt1 released more total VOCs than did 5422 and 5422CBCL; and 5422Bt1 emitted more amounts of linalool, DMNT, and (*E*)-β-farnesene than did 5422 and 5422CBCL (**Figure 1C**). In addition, intact plants of the three cultivars released a few compounds, e.g., linalool, DMNT and (*E*)-β-farnesene, in trace amounts (**Supplementary File 2**).

TABLE 1 | The attractiveness of the *Bt* maize and regular maize plants to the egg parasitoid.

	Treatments	Wasp preference (%)	Wasp response (%)	*P*-value (two-tailed *t*-test)*
Intact plants compared to an empty arm (blank control)	5422	55.3	59.4	0.275
	Empty arm	44.7		
	5422Bt1	56.1	64	0.172
	Empty arm	43.9		
	5422CBCL	52.8	56.3	0.348
	Empty arm	47.2		
Regurgitant treated plants compared to an empty arm (blank control)	5422	71.4	76.6	**<0.001**
	Empty arm	28.6		
	5422Bt1	68.6	79.7	**<0.001**
	Empty arm	31.4		
	5422CBCL	76.6	73.4	**<0.001**
	Empty arm	23.4		
JA treated plants compared to an empty arm (blank control)	5422	79.5	68.8	**<0.001**
	Empty arm	20.5		
	5422Bt1	72.9	75	**0.005**
	Empty arm	27.1		
	5422CBCL	71.7	71.9	**<0.001**
	Empty arm	28.3		
Comparisons between intact plants of different cultivars	5422	55.6	56.3	0.524
	5422Bt1	44.4		
	5422	55.0	62.5	0.11
	5422CBCL	45.0		
	5422Bt1	52.6	59.4	0.696
	5422CBCL	47.4		
Comparisons between regurgitant treated plants of different cultivars	5422	55.6	84.4	0.787
	5422Bt1	44.4		
	5422	53.7	84.4	0.155
	5422CBCL	46.3		
	5422Bt1	50.0	75.0	1
	5422CBCL	50.0		
Comparisons between JA treated plants of different cultivars	5422	51.9	84.4	0.773
	5422Bt1	48.1		
	5422	51.7	90.6	0.661
	5422CBCL	48.3		
	5422Bt1	48.3	90.6	0.617
	5422CBCL	51.7		

**The bold P values indicate significant statistical differences ($P < 0.05$) between treatments.*

DISCUSSION

Genetic Transformations Sometimes Change Induced VOCs Emissions of Plants and the Possible Mechanism

In this study, we found the 5422Bt1 released a few terpenes in a higher amount than did its nearly isogenic non-*Bt* maize when the plants were treated by caterpillar regurgitant or JA. Although a few studies reported that introduction of new genes into plants did not change the VOCs emission (Dean and De Moraes, 2006; Sun et al., 2013; Liu et al., 2015), some studies confirmed that the VOCs emissions of GM plants were changed quantitatively compared to that of their regular isolines. For example, the *Bt* maize N4640Bt (event Bt11) emitted several VOCs in a smaller amount than did its isogenic non-*Bt* line when the plants were damaged by mechanical wounds and then applied with caterpillar regurgitant (Turlings et al., 2005). When infested by the leafminer species *Phyllonorycter blancardella* (Fabricius), the GM apple plants with a scab resistance gene emitted less (*E,E*)-α-farnesene than did their conventional equivalents (Vogler et al., 2010). A transgenic soybean cultivar expressing a glyphosate-resistant gene released a few volatile compounds in a higher amount than

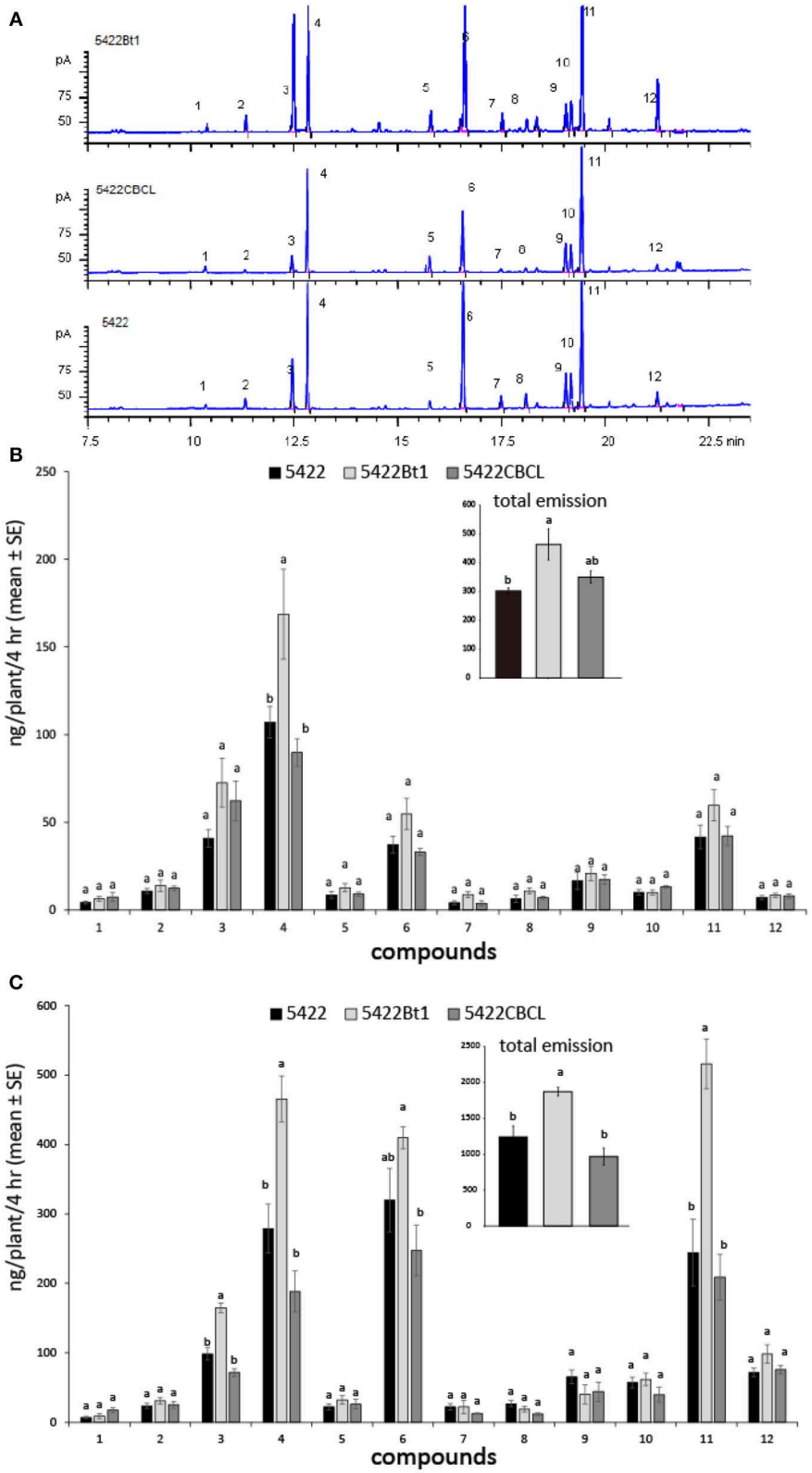

FIGURE 1 | Volatile emissions of the *Bt* maize and the non-*Bt* maize lines under different treatments. **(A)** The chromatographs of maize seedlings induced by JA solution. The compounds were 1 = (*Z*)-3-hexen-1-yl acetate; 2 = (*E*)-β-ocimene; 3 = linalool; 4 = (3*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT); 5 = phenethyl acetate; *(Continued)*

FIGURE 1 | 6 = indole; 7 = methyl anthranilate; 8 = geranyl acetate; 9 = (*E*)-β-caryophyllene; 10 = (*E*)-α-bergamotene; 11 = (*E*)-β-farnesene; 12 = (*E*)-nerolidol. **(B)** The volatile emissions ($N = 4$) of the three cultivars when the plants were mechanically damaged and then treated by caterpillar regurgitant. **(C)** The volatile emissions ($N = 4$) of the three cultivars when the plants were treated by JA. A one-way ANOVA with the Holm-Sidak *post hoc* analysis was used for pairwise comparisons, and letters on the bar figures indicated statistical differences ($P < 0.05$). The details of statistical results and data were presented in **Supplementary Files 1**, **2**, respectively.

did its conventional isoline when the plants were damaged by the soybean looper *Chrysodeixis includes* (Walker) or the velvetbean caterpillar *Anticarsia gemmatalis* Hübner (Strapasson et al., 2016a,b). As a result, the herbivore-damaged GM soybean plants were more attractive to the larval parasitoid *Meteorus rubens* (Nees) than the conventional isoline (Strapasson et al., 2016a).

The mechanism of why some transformations of foreign genes in crops change some of their metabolites is unknown. However, some hypotheses have been proposed to explain different cases. For example, the quantitative modifications of inducible secondary metabolites of transgenic plants are possibly due to unintended changes on resource allocation by continuous biosynthesis of other proteins, such as *Bt*. As a result, the *Bt* maize line more likely releases several volatile compounds in a lower amount than does its non-*Bt* isoline due to deficiency of resources when the plants are induced by caterpillar regurgitant (Turlings et al., 2005). However, after genetic transformations, some inducible VOCs, such as terpenoids, have actually increased (Strapasson et al., 2016b). One possible explanation is that the newly biosynthesized protein (responsible for herbicide tolerance in this case) has affected or is involved in the plant hormone-mediated defensive pathway, which responds to produce some VOCs (Strapasson et al., 2016a). Therefore, genetic transformations possibly lead to quantitative differences in some VOCs emissions, or some other required metabolites, such as amino acids in plant phloem reported by Faria et al. (2007). The molecular mechanism needs further investigations.

The Variation of VOCs Emission Caused by Genetic Transformations Still Fall Within the Variability Among Conventional Cultivars

The quantitative changes of induced VOCs emission that resulted from genetic transformations have been evaluated by comparing them with those from conventional cultivars. The changes caused by genetic modifications in maize and apple plants are relatively small compared to those that result from traditional breeding programs (Turlings et al., 2005; Vogler et al., 2009, 2010). For example, upon the herbivory by leafminers on apple trees, some traditional cultivars released a different amount of a key terpenoid volatile compound that attracted parasitoids, but a GM cultivar and its regular isoline emitted similar amounts of the compound, suggesting that the alterations of leaf chemistry are more apparent between conventional cultivars (Vogler et al., 2009). In maize, after analyzing 31 conventional maize lines, Degen et al. (2004) found that the variations of the total emission of induced VOCs were enormously huge, with up to a 70 times difference between two extreme lines. Some genotypes even did not produce an important terpenoid compound (*E*)-β-caryophyllene after receiving the treatment of mechanical wounds and caterpillar regurgitant (Gouinguené et al., 2001; Degen et al., 2004). The compound was reported to be a key compound involved in tritrophic interactions in the soil environment (Rasmann et al., 2005). The 5422Bt1 maize released VOCs by about 50% higher than its non-*Bt* isoline 5422. This discrepancy is probably smaller than that between many regular maize cultivars.

Transformation of *Bt* Genes Does Not Influence the Attractiveness to Parasitoids

In our study, we found that expressing *Bt* proteins in maize plants did not affect their attractiveness to the egg parasitoid *T. ostriniae*. Egg parasitoids possibly use many kinds of volatile cues to exploit their hosts, such as host pheromones, egg odors, host frass smells or oviposition-induced plant volatiles (OIPVs) (Meiners and Hilker, 1997; Fatouros et al., 2005, 2008; Hilker and Fatouros, 2015). In addition, HIPVs are attractive to many egg parasitoids of, for example, lepidopteran and hemipteran herbivores (Reddy et al., 2002; Lou et al., 2005; Moraes et al., 2005; Williams et al., 2008; Peñaflor et al., 2011b; Tamiru et al., 2011; Michereff et al., 2019). Caterpillar-damaged plants are reported to be attractive to some *Trichogramma* spp. (Peñaflor et al., 2011b; War et al., 2016). Attraction to HIPVs is possibly important for some generalist *Trichogramma* parasitoids, when their host eggs and larvae co-occur (Peñaflor et al., 2011b; Michereff et al., 2019). Importantly, the volatile cues such as host pheromones, egg smells or even OIPVs are probably released with relatively smaller amounts compared to HIPVs, and then more likely working in a relatively short range (Peñaflor et al., 2011a; Michereff et al., 2016; Xu and Turlings, 2018). Therefore, HIPVs possibly facilitate host locations for some egg parasitoids in different ways.

Our data are in line with those that have been derived from the studies of some parasitoid species in rice (Liu et al., 2015), cotton (Moraes et al., 2011; Yao et al., 2016), and oilseed rape plants (Schuler et al., 1999, 2003). In maize, under treatment of caterpillar regurgitant, the *Bt* plant (N4640Bt) emitted fewer amounts of several volatile compounds than did its isogenic non-*Bt* line, but they showed similar attractiveness to two larval parasitoid species (Turlings et al., 2005).

Two major classes of HIPVs, green leaf volatiles and terpenoids, have often been considered to recruit natural enemies because they are the most abundant compounds emitted from plants after herbivore attack (Gershenzon and Dudareva, 2007; Arimura et al., 2009). However, some important volatile(s) emitted by maize plants responsible for attracting parasitoids are still not identified (D'Alessandro and Turlings, 2006; Turlings and Erb, 2018), though much effort has been made to identify them. For example, in maize plants, the attractiveness of HIPVs to the larval parasitoid *Cotesia marginiventris* (Cresson)

probably relies on a combination of certain polar and non-polar compounds and the polar compounds are more important than the non-polar ones (D'Alessandro et al., 2009). However, the key attractants are normally released in trace amounts and even below the detection threshold of the GC analysis (Gouinguené et al., 2005; D'Alessandro et al., 2009). In contrast, some components of HIPVs emitted in large amounts (such as indole and some sesquiterpenes) do not attract natural enemies of herbivores (D'Alessandro et al., 2009; von Mérey et al., 2011). Those studies help us understand that in spite of occasionally changing the emitting amounts of some common VOCs in maize plants by transgenic events, the main attractiveness to parasitoids is possibly unchanged.

In conclusion, transformations of foreign genes to crops may change their VOCs emission. However, the variations are normally less apparent than those among conventional cultivars. Importantly, the modifications of VOCs emission normally do not reduce the attractiveness of GM plants to natural enemies. The findings indicate that releasing natural enemies is still likely to be an effective way to control non-target or *Bt*-resistant pests in *Bt* fields.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

JW, GC, BT, and HX conceived and designed the research. XW, GC, BT, and HX conducted experiments. GC, BT, and HX analyzed the data. HX wrote a first draft. All authors commented on the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2019.00160/full#supplementary-material

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Regulatory Status of Genome-Edited Organisms Under the Japanese Cartagena Act

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The Japanese government recognizes the substantial values of genome-edited agricultural organisms and has defined in which cases these are covered by the existing regulatory framework to handle this technology. Genome-editing technologies could revolutionize and accelerate plant breeding owing to the simplicity of the methods and precision of genome modifications. These technologies have spread rapidly and widely, and various genome-edited crops have been developed recently. The regulatory status of genome-edited end products is a subject of controversy worldwide. In February 2019, the Japanese government defined genome-edited end products derived by modifications of SDN-1 type (directed mutation without using a DNA sequence template) as not representing "living modified organisms" according to the Japanese Cartagena Act. Here, we describe the classification and regulatory status of genome-edited end products in this decision. We hope that reporting the progress in Japan toward the implementation of this regulatory approach will provide insight for scientific and regulatory communities worldwide.

Keywords: genome editing, regulatory status, Japan, Cartagena Protocol, LMOs

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INTRODUCTION

Article 8 (g) of the Convention on Biological Diversity (CBD)[1] establishes the obligation to Parties to "establish or maintain means to regulate, manage or control the risks associated with the use and release of living modified organisms (LMOs)." Building on that, Article 1 of the Cartagena Protocol on Biosafety (CPB) aims "to contribute to ensuring an adequate level of protection in the field of the safe transfer, handling and use of living modified organisms resulting from modern biotechnology that may have adverse effects on the conservation and sustainable use of biological diversity, taking also into account risks to human health, and specifically focusing on transboundary movements."[2] According to the general provisions in Article 2 of the Protocol,[2] each party shall take necessary and appropriate legal, administrative, and other measures to implement its obligations under this Protocol. Because the Cartagena Protocol was established in awareness of future technological developments, it has to be considered to what extent it applies to organisms derived by genome-editing techniques.

[1]Convention on Biological Diversity (CBD). *Article 8, in situ Conservation*. Available online at: https://www.cbd.int/convention/articles/default.shtml?a=cbd-08 (accessed October 8, 2019).

[2]CBD. *Text of the Cartagena Protocol on Biosafety*. Available online at: https://bch.cbd.int/protocol/text (accessed October 8, 2019).

Since the Eighth meeting of the Conference of the Parties serving as the meeting of the Parties to the Cartagena Protocol (COP/MOP-8) in 2016[3], genome editing has become a major focus. At COP14, it was agreed that "broad and regular horizon scanning, monitoring and assessing of the most recent technological developments is needed ["taking into account that this may include genome editing"] for reviewing new information regarding the potential positive and potential negative impacts of synthetic biology *vis-à-vis* the three objectives of the Convention and those of the Cartagena Protocol and Nagoya Protocol"[4].

Japan is a Party to the Cartagena Protocol. In 2003, the domestic "Act on the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms" for implementing the Cartagena Protocol (called the Cartagena Act) was established[5]. The Japanese government has been proactively looking at the organisms of genome editing, and on February 8, 2019[6], decided that some genome-edited organisms should be considered as LMOs while others are not subject to the Cartagena Act. No announcement of the publication of this notice has been made in any foreign language except for a short English flier by the Ministry of the Environment (MOE)[7], so here we would like to analyze and explain its content for an international audience. In general, Japan has been rather slow in implementing the regulatory framework on biotechnology (Watanabe et al., 2004), and the present notification on genome editing may facilitate more timely development of commercial products. This interpretation of the regulatory framework in Japan could encourage other countries to consider similar balanced legislation.

CURRENT REGULATORY STATUS OF GENOME-EDITED ORGANISMS WORLDWIDE

The Organization for Economic Co-operation and Development (OECD) Working Group for the Harmonization of Regulatory Oversight in Biotechnology has discussed the safety and regulatory considerations raised by genome-edited organisms. In June 2018, "The OECD Conference on Genome Editing: Applications in Agriculture—Implications for Health, Environment and Regulation" was held in Paris[8].

Organisms developed through new breeding techniques, including genome editing, may contain nucleic acids from a foreign source. The regulatory status of genome-edited organisms has been discussed in various countries, and the regulatory approaches differ across countries. On March 28, 2018, the U.S. Secretary of Agriculture Sonny Perdue stated that the U.S. Department of Agriculture (USDA) "does not regulate or have any plans to regulate plants that could otherwise have been developed through traditional breeding techniques, as long as they are not plant pests or developed using plant pests"[9]. In Argentina (Whelan and Lema, 2015), Chile (Cameron et al., 2017), and Brazil (Chandrasekaran et al., 2016), the status of organisms obtained through new plant breeding techniques requires confirmation that they have no nucleic acids derived from foreign organisms.

On July 25, 2018, the Court of Justice of the European Union issued its judgment that "organisms obtained by means of techniques/methods of mutagenesis constitute GMOs within the meaning of that provision" and "only organisms obtained by means of techniques/methods of mutagenesis which have conventionally been used in a number of applications and have a long safety record are excluded from the scope of that directive" under the directive 2001/18/EC (Official Journal of the European Communities, 2001).This ruling has resulted in much uncertainty and discussion regarding the regulatory status of genome-edited organisms in general[10].

Two countries in Oceania have different regulations. Australia gave notice of "Gene Technology Amendment (2019 Measures No. 1) Regulations 2019," which is modified law of "The Gene Technology Act 2000" on April 4, 2019[11]. The Australian government will not regulate the use of gene-editing techniques in plants, animals, and human cell lines that do not introduce a novel combination of genetic material (Mallapaty, 2019). According to Fritsche et al. (2018), "in 2014, New Zealand's Environmental Protection Authority ruled that plants produced via genome-editing methods, where no foreign DNA remained in the edited plant, would not be regulated as LMOs," but this decision was overturned by the High Court; currently, New Zealand considers all gene-edited organisms as LMOs.

[3]CBD. *Outline of Guidance on Risk Assessment Of Living Modified Organisms Developed Through Synthetic Biology, UNEP/CBD/BS/COP-MOP/8/8/ADD3*. Available online at: https://www.cbd.int/doc/meetings/bs/mop-08/official/bs-mop-08-08-add3-en.pdf (accessed October 8, 2019).

[4]CBD. *Synthetic Biology, Draft Decision Submitted by the Chair of Working Group II, CBD/COP/14/WG.2/CRP.20.* Available online at: https://www.cbd.int/doc/c/043c/a200/78251e44a6f7ceed13b44312/cop-14-wg-02-crp-20-en.pdf (accessed October 8, 2019).

[5]Japan Biosafety Clearing House (J-BCH). *Domestic Law and Regulations.* Available online at: https://www.biodic.go.jp/bch/english/law.html (accessed October 8, 2019).

[6]Ministry of the Environment (MOE). *About the Handling of Organisms Produced by the Use of Genome Editing Technology that Do Not Match the Definition of "Genetically Modified Organisms" in the Cartagena Act.* Available online at: http://www.biodic.go.jp/bch/download/genome/genome_tsuuchi20190208.pdf (accessed June 14, 2019).

[7]MOE. *To Genome Editing Technologies Users*. Available online at: https://www.env.go.jp/press/2_2_%20genome%20editing_En.pdf (accessed July 18, 2019).

[8]OECD. *The OECD Genome Editing Hub, OECD Conference on Genome Editing: Applications in Agriculture on 28–29 June 2018.* Available online at: http://www.oecd.org/environment/genome-editing-agriculture/ (accessed June 14, 2019).

[9]U.S. Department of Agriculture (2018). *Secretary Perdue Issues USDA Statement on Plant Breeding Innovation.* Washington, DC. Available online at: https://www.usda.gov/media/press-releases/2018/03/28/secretary-perdue-issues-usda-statement-plant-breeding-innovation (accessed June 14, 2019).

[10]Court of Justice of the European Union. *Judgement of the Court in Case C-528/16: Court of Justice of the European Union.* Available online at: http://curia.europa.eu/juris/document/document.jsf?text=&docid=204387&pageIndex=0&doclang=EN&mode=lst&dir=&occ=first&part=1&cid=138460 (accessed October 8, 2019).

[11]Federal Register of Legislation. *Gene Technology Amendment (2019 Measures No. 1) Regulations 2019.* Available online at: https://www.legislation.gov.au/Details/F2019L00573 (accessed July 31, 2019).

HISTORY OF DISCUSSION ON REGULATORY STATUS OF GENOME-EDITED ORGANISMS IN JAPAN

Under the Cartagena Act, the use of living modified crops requires reviews of the environmental risk to biodiversity associated with the deliberate release of such crops. The Cartagena Act states that LMOs are regulated in terms of the final products as "living organisms having nucleic acids obtained by utilizing a technique for processing nucleic acids outside the cell for the purpose of transferring or replicating the nucleic acids by transferring them into a cell, virus, or viroid" (Chapter I, Article 2, item 2)[5] in accordance with items (g) and (h) of the "Use of Terms" of Article 3 of the CPB[2]. An LMO is any organism with inserted extracellularly processed nucleic acid (including RNA)[7]. If the end products of genome-editing technology have no remnants of inserted nucleic acid or its replicated product and are undistinguishable from those developed *via* traditional breeding methods, they are not LMOs. In the Cartagena Act, a "replicated product" is replicated nucleic acid from transformed nucleic acid that is neither RNA nor protein.

In Japan, the regulatory perspective of genome-edited end products has been discussed over the past 5 years. In August 2014, the Science Council of Japan released the report "Current status and problems of new plant breeding technology (NPBT)"[12]. This report stated that knowledge accumulation and management operations according to the Cartagena Act are important for crop development using NPBT. In September 2015, the New Plant Breeding Technique Study Group, established at the Secretariat of Agriculture, Forestry and Fisheries Research Council, the Ministry of Agriculture, Forestry and Fisheries (MAFF), released the document "Toward the development and practical application of crops using new plant breeding techniques (NPBTs) such as genome editing"[13], which asserted that appropriate measures will be implemented under the Cartagena Act for dealing with living organisms with foreign genes transiently introduced during breeding, and international harmonization on regulatory status will be promoted. In August 2016, at the Expert Committee on LMOs of the Nature Conservation Committee, the Central Environment Council, MOE issued a report entitled "Examining enforcement of the Cartagena Act," which stated that decision making on regulatory status of organisms that do not contain exogenous nucleic acids created by new breeding techniques such as genome editing is an urgent issue, and it is necessary to carefully consider this status in light of the latest scientific knowledge and international harmonization[14]. In September 2016, a member of the House of Councilors submitted the document "Subjective Questionnaire on Genetic Research, Development, and Regulation of Genome Editing Technology" to the Cabinet Office[15]. The Council of Science, Technology and Innovation of the Cabinet Office has established the Working Group for Bio-Strategy, and the interim report of the Working Group was issued in June 2018[16]. This report suggested that clarification of the regulatory status of genome-edited crops under the Cartagena Act and Food Sanitation Act is at an early stage, and promotion of public understanding of genome-editing techniques is needed. Later in the same month, the Cabinet Office endorsed the Integrated Innovation Strategy[17], which stated that the regulatory status of organisms obtained by genome editing in line with the Cartagena Act and the regulatory status of agricultural and fishery organisms obtained using this technology under the Food Sanitation Act should be clarified by the end of fiscal year 2018, and efforts should be made to promote international harmonization. On July 11, 2018, The Expert Meeting on Genome Editing Technologies under the Cartagena Act was established within the Expert Committee on LMOs of the Nature Conservation Committee, the Central Environment Council, MOE as the administration of the Cartagena Act[18]. On August 7, the meeting was held for the first time to discuss the regulatory status of genome-editing technology under the Cartagena Act[19]; on August 20, the second meeting summarized the discussion on the regulatory classification and status of genome-editing technology as a draft report[20]. On August 30, the 2nd Expert Committee on LMOs produced the draft report entitled "Classification and status of organisms produced by application of genome-editing technology under the Cartagena Act"[21]. In 2018 (September 20–October 19), a public consultation on the proposal was arranged[22]. On January 21, 2019, the feedback was discussed at the Nature Conservation

[12]Science Council of Japan. *Current Status and Problems of New Plant Breeding Technology (NPBT).* Available online at: http://www.scj.go.jp/ja/info/kohyo/pdf/kohyo-22-h140826.pdf (accessed June 14, 2019).

[13]New Plant Breeding Technique Study Group (2015). *Toward the Development and Practical Application of Crops Using New Plant Breeding Techniques (NPBTs) Such as Genome Editing.* Available online at: http://www.affrc.maff.go.jp/docs/commitee/nbt/attach/pdf/top-2.pdf (accessed June 14, 2019).

[14]MOE. *Minute of the Expert Committee on LMOs (Third in Fiscal Year 2016).* Available online at: https://www.env.go.jp/council/12nature/y127-03a.html (accessed June 14, 2019).

[15]House of Councilors, The National Diet of Japan. *Subjective Questionnaire on Genetic Research, Development, and Regulation of Genome Editing Technology of the 192nd Diet.* Available online at: http://www.sangiin.go.jp/japanese/joho1/kousei/syuisyo/192/meisai/m192004.htm (accessed June 14, 2019).

[16]Cabinet Office. *Working Group for Bio-Strategy and the Interim Report.* Available online at: https://www8.cao.go.jp/cstp/tyousakai/bio/bio_chukan.pdf (accessed June 14, 2019).

[17]Cabinet Office. *The Integrated Innovation Strategy.* Available online at: http://www8.cao.go.jp/cstp/senryakukaigi/3kai/siryo3.pdf (accessed June 14, 2019).

[18]MOE. *Minute of the Expert Committee on LMOs (First in Fiscal Year 2018).* Available online at: https://www.env.go.jp/council/12nature/02_3.html (accessed June 14, 2019).

[19]MOE. *Minute of the Expert Meeting on Genome Editing Technologies under the Cartagena Act (First in Fiscal Year 2018).* Available online at: https://www.env.go.jp/council/12nature/post_56.html (accessed June 14, 2019).

[20]MOE. *Minute of the Expert Meeting on Genome Editing Technologies under the Cartagena Act (Second in Fiscal Year 2018).* Available online at: https://www.env.go.jp/council/12nature/30_3.html (accessed June 14, 2019).

[21]MOE. *Minute of the Expert Committee on LMOs (Second in Fiscal Year 2018).* Available online at: https://www.env.go.jp/council/12nature/30_10.html (accessed July 31, 2019).

[22]MOE (2018). *Call for Public Comments on "Classification and Handling of Organisms Produced by Application of Genome Editing Technology under the Cartagena Act."* Available online at: https://www.env.go.jp/press/105960.html (accessed June 14, 2019).

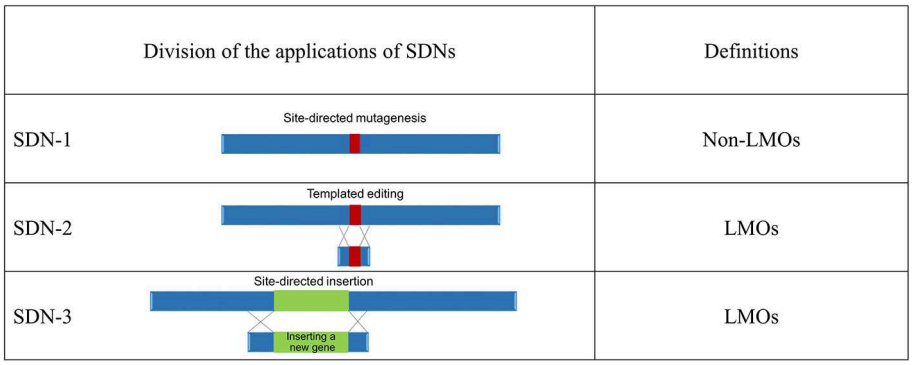

FIGURE 1 | Regulatory overview of genome-edited organisms in Japan. Ministry of the Environment presented a preliminary draft[21] to define organisms produced using three applications of site-directed nucleases (SDNs). Living modified organisms (LMOs): any living organism that possesses a novel combination of genetic material obtained through the use of modern biotechnology[2].

Committee, the Central Environment Council[23], and on February 8, 2019, the final decision was reported by the MOE[6]. Here, we report the key elements of the final decision made by the MOE.

REGULATORY STATUS OF GENOME-EDITED ORGANISMS IN JAPAN

Genome-editing techniques are classified into three principal categories, site-directed nuclease (SDN)-1, that is, site-directed mutagenesis, SDN-2, that is, templated editing, and SDN-3, that is, site-directed gene insertion (**Figure 1**). This categorization is based on the definition by Lusser et al. (2011, 2012). The types of artificial nucleases, which include zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR), used for targeted modification (Podevin et al., 2013) are considered.

The end products from the SDN-1 methods do not contain inserted nucleic acid or its replicated product, so they do not satisfy the definition of LMOs in the Cartagena Act (Chapter I, Article 2, item 2)[5]. On the other hand, the end products obtained by the SDN-2 and SDN-3 methods contain inserted nucleic acids processed extracellularly and are categorized as LMOs. This categorization is the same as in a document issued by the Australian Government[24]. The size of the nucleic acid insert is undefined in the Cartagena Act. Any organism with inserted extracellularly processed nucleic acid (including RNA) is regarded as an LMO and is subject to the regulations stipulated in the Cartagena Act unless the complete removal of the inserted nucleic acid (including RNA) or its replicated product is confirmed. The final determination according to the MOE[6] approach would be applicable to null segregants, in which the inserted foreign gene is segregated out through backcrossing.

In the future, the newly developed biotechnological end products have to be thoroughly classified in terms of whether or not they contain extracellularly processed nucleic acids. Technology users are requested to notify the government with information on unregulated end products created through genome-editing technology, including the details of their production and any knowledge of their impact on biodiversity[4] prior to use. Competent national authorities [administrative agencies, such as the MAFF, the MOE, and the Ministry of Education, Culture, Sports, Science and Technology (MEXT)] call on users of genome-editing SDN-1-based technologies to submit a review of the biological characteristics and impact on biodiversity of genome-edited organisms to the appropriate ministry. Submission is not needed if there has been no change to a previously submitted review, or genome-edited organisms are used in an environment in which containment measures have been taken.

In the case of a probable risk to biodiversity, the competent national authority will require additional information from the user; then, necessary measures can be taken. MOE will post-annually some information on unregulated end products, mainly the taxonomical species of the modified organism, change of traits added by the modification, usage of the organism, and discussion on possible influences on biological diversity when the organism is used; all information to be provided is listed in a flyer in English produced by the MOE[7], and the names of the administrative agency to notify depending on the use of the organism, on the website[6,7]. In case of any concern about the impact of a genome-edited organism on biodiversity, the user must take necessary measures to mitigate the effect on biodiversity immediately according to the Cartagena Act and promptly report this to the administrative agencies in charge, which would take appropriate measures in consideration of the public policy on biodiversity

[23]MOE (2019). *The Nature Conservation Committee, the Central Environment Council. (37th).* Available online at: https://www.env.go.jp/council/12nature/37_3.html (accessed July 31, 2019).

[24]Office of the Gene Technology Regulator. *Technical Review of the Gene Technology Regulations 2001-2016 Discussion Paper Consultation.* Available online at: http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/reviewdiscussionpaper-htm (accessed July 18, 2019).

conservation. The administrative agencies can also require additional information upon considering the characteristics of the species.

TOWARD FUTURE DECISION MAKING ON GENOME-EDITED ORGANISMS IN JAPAN

At first, in response to the draft by the MOE[21], the Japanese Society of Breeding made a statement on October 1, 2018[25]. The Society appreciated that users are requested to provide information on genome-edited organisms that are not subject to the Cartagena Act. The Society stated that if this proposed policy enters Japanese legislation, breeding institutions, universities, and seed companies can make substantial contributions to the stable supply of food through the improvement of plants using genome-editing technology. The administrative agencies such as MAFF, MOE, MEXT, and the Ministry of Health, Labor, and Welfare should work together to clarify procedures for providing information for the use of genome-edited organisms. These regulations will promote practical use of superior crop varieties generated through genome-editing technology.

Although the latest Japanese government regulation was noticed on February 8, 2019[6], the scientific aspects, such as the method for assessing the persistence of a foreign gene region in a null segregant and the effects of unintentional mutations including off-target effects, need to be clarified. A method has been established for confirming the persistence of a foreign DNA fragment by using a next-generation sequencer and improved Southern hybridization, which is outlined in Tabei (2019). The current methods for detection of DNA sequence alterations through genome-editing techniques were summarized in the European Network of GMO Laboratories[26], and appropriate judgment criteria and detection methods are being discussed worldwide.

[25] Japanese Society of Breeding. *A Statement from the Japanese Society of Breeding in Response to the Call for Public Comments on "Classification and Handling of Organisms Produced by Application of Genome Editing Technology under the Cartagena Act."* Available online at: https://www.nacos.com/jsb/02/02PDF/20181001_JSBseimei.pdf (accessed June 14, 2019).

[26] European Network of GMO Laboratories (ENGL) (2019). *Detection of Food and Feed Plant Products Obtained by New Mutagenesis Techniques (JRC116289).* Available online at: http://gmo-crl.jrc.ec.europa.eu/doc/JRC116289-GE-report-ENGL.pdf (accessed October 8, 2019).

CONCLUSIONS AND RECOMMENDATIONS

Japan has decided on rules for regulatory status of genome-edited organisms[6]. The organisms produced by SDN-1 are not subject to regulation under the Cartagena Act, as they are considered similar to those produced by conventional breeding technologies. Although mutations in the organisms produced by SDN-2 are equivalent to those that occur naturally, such organisms are considered LMOs under the Cartagena Act if they possess inserted extracellularly processed nucleic acid. The regulatory status of organisms produced by SDN-2 is considered on a case-by-case basis worldwide. Organisms produced by SDN-3 are considered LMOs. The decision of the MOE of Japan makes it possible for each stakeholder to judge the actions needed on the basis of defined criteria. We hope that the availability of this information will promote the use of genome editing for plant breeding under the proper regulatory status of the Cartagena Act in Japan and will be helpful for future discussions at the OECD and regulatory decision making in other countries.

AUTHOR CONTRIBUTIONS

MT organized the manuscript. KW provided input on international legal instruments. RO verified the information on domestic regimes. All authors discussed the results and contributed to the final manuscript.

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Regulation of GM Organisms for Invasive Species Control

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Invasive species can cause significant harm to the environment, agriculture, and human health, but there are often very limited tools available to control their populations. Gene drives (GD) have been proposed as a new tool which could be used to control or eliminate such species. Here, GD describes a variety of molecular biology applications which all enable the introduction of genetic elements at a higher than expected frequency. These elements can change the genotypes in target populations rapidly with consequences either for (intrinsic) fitness or host-parasite interaction, or both. Beneficial applications are foreseen for human and animal health, agriculture, or nature conservation. This rapidly developing technology is likely to have major impacts in the fight against various diseases, pests, and invasive species. The majority of GD applications involve genetic engineering and novel traits. Therefore, applicants and GMO regulators need to interact to achieve the benefits in innovation while cautiously avoiding unacceptable risks. The release into the environment may include transboundary movement and replacement of target populations, with potential impact on human/animal health and the environment. This article summarizes knowledge-based discussions to identify information gaps and analyzes scenarios for responsible introduction of GD organisms into the environment. It aims to connect the latest scientific developments with regulatory approaches and decision-making.

Keywords: genome editing, gene drive, environmental risk assessment (ERA), regulation, invasive species

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INTRODUCTION

Impacts of Invasive Species

Invasive species are animals and plants introduced accidentally or deliberately into a natural environment different from the one they originate, with serious negative consequences for their new environment. This definition was taken from a recent JRC report (Tsiamis et al., 2019), which lists such invasive species of EU concern. Invasive species include viruses, microorganisms, fungi, insects, and other invertebrates, feral animals, marine pests, and weedy plants. Invasive species have caused serious adverse effects to human health, agriculture, and the environment. For example, the high rate of extinction of Australian land mammals (>10% of the 273 endemic terrestrial species over the last ~200 years) is likely due primarily to predation by invasive species, particularly feral cats (*Felis catus*) and European red foxes (*Vulpes vulpes*) (Woinarski et al., 2015; Murphy et al., 2019).

For humans, one of the most dangerous effects of invasive species is their direct pathogenic effects or indirect vector activity for disease. In Europe, the Asian tiger mosquito (*Aedes albopictus*)

is thought to have been accidently imported from Southern China on recycled tyres and lucky bamboo plants (*Dracaena sanderiana*). It vectors many pathogens, including yellow fever and chikungunya virus (Medlock et al., 2012).

Invasive plants impact the environment (for instance) by outcompeting native plants and reducing agricultural production. But they may also negatively impact human health. Ragweed (*Ambrosia artemisiifolia* L) came to Europe from North America as a contaminant in bird seed. It has spread rapidly and produces highly allergenic pollen that causes hay fever in 4–5% of Europeans (Richter et al., 2013).

These invasive species also cause high economic damages or losses. For example, in Australia the annual cost of pest animals was estimated at $597M in 2013–14 in lost productivity and cost of controls (McLeod, 2016). Similarly, weeds were estimated to cost nearly $5 billion across Australia in 2018. The costs of chemical control in broad acre cropping and lost production costs in the grain, beef and wool industries lead to most of these impacts and damages (McLeod, 2018).

Control or eradication of invasive species once they have established is difficult. Weed and pest control managers need a variety of tools to use in integrated pest management approaches (Messing and Wright, 2006). A number of these biocontrol tools including sterile-release, YY Males, Trojan Female Technique and gene drive were reviewed at the Genetic Biocontrol for Invasive Species Workshop in Tarragona Spain, March 31st, 2019, which was sponsored by the OECD Co-operative Research Programme: Biological Resource Management for Sustainable Agricultural Systems (CRP). The workshop raised awareness of benefits and risks of invasive species control in general, with GD techniques as a case example. The meeting provided the opportunity for an open exchange of views. A summary of the technical and historical developments in this emerging field is presented by Teem et al. (in preparation). The present article highlights regulatory approaches and decision-making for invasive species control including GD.

Gene Drive (GD) as a Specific Case for Both Introduction and Control of Invasive Species

Gene Drive (GD) describes a variety of molecular biology applications which all enable the introduction of genetic elements that are inherited at frequencies above those predicted by the Mendelian rules that means the transmission of a specific allele to the next generation is greater than the expected 50%. GDs only work in out-crossing sexually reproducing species as they are active in the germline or when the embryo is formed. Gene drives can theoretically spread through the entire population of a species or, depending on the sequence targeted, could be limited to certain areas or populations. However, cage experiments with insects and computer modeling have shown that some gene drives may not spread unchecked through target populations due to the evolution of resistance (KaramiNejadRanjbar et al., 2018) or negative effects on fitness in the target species (de Jong, 2017).

Different types of gene drives occur naturally in a number of species. Meiotic drives have been reported in insects and plants, for example in *Drosophila melanogaster* (McDermott and Noor, 2010) and *Silene latifolia* (Taylor and Ingvarsson, 2003); cytoplasmic incompatibility caused by *Wolbachia* bacteria (Sinkins, 2004) and maternal-effect dominant embryonic arrest (Medea) in flour beetle (*Tribolium castaneum*) (Beeman et al., 1992; Rüdelsheim and Smets, 2018).

An example of how natural gene drives can be utilized to control invasive species is that of *Wolbachia* endosymbiont bacteria (**Box 1**).

BOX 1 | Example of non-GMO gene drive—Regulation of *Wolbachia* containing insects

Wolbachia are bacteria which infect a wide range of arthropod hosts and manipulate the host reproduction (Sinkins, 2004). They generate a gene drive by causing incompatibility between eggs and sperm or by killing of males. The bacteria are maternally inherited and their manipulation of reproduction favors survival of infected females.

Wolbachia pipientis has been introduced into *Aedes aegypti* populations where they greatly reduce the replication of dengue virus and other human pathogens within the infected mosquito (Kambris et al., 2009; Moreira et al., 2009; Bian et al., 2010; Schmidt et al., 2017). As transfer of a whole organism is not considered to lead to a GMO, field trials of this work are regulated by Australian Pesticides and Veterinary Medicines Authority under the *Agricultural and Veterinary Chemicals Code Act 1994* as a veterinary medical product (De Barro et al., 2011).

New molecular techniques enable a previously unachievable spatially and functionally precise modification of the genomes of plants, animals, and microorganisms. These techniques enable a range of changes from site-specific alterations of single nucleotides, to the site-specific insertion of entire genes. Currently, attention is focused on CRISPR Cas9 technology, but many other naturally occurring site specific nucleases can also be used. Engineered gene drives introduce genetic changes with the help of natural components of a gene drive or site specific nucleases. The consequence is a rapid increase of the modified genes in the organism's population.

Internationally, there is rapidly growing research interest in using gene drives for the control of a variety of invasive species. Potential applications include:

- Controlling populations of invasive animals (e.g., exotic rodents), to protect natural environments (Leitschuh et al., 2018; Moro et al., 2018; Harvey-Samuel et al., 2019)
- Controlling invasive plants (weeds) of natural or agricultural environments (Neve, 2018)

Use of GD for control of invasive species, diseases and pests may offer great benefits to society. However, as with any technology for species control, it may also pose risks to wild species or ecosystems. GD raises new challenges for regulation, specifically when the GD involves genetically modified organisms (GMO). GMOs are regulated in most countries, and are also covered by

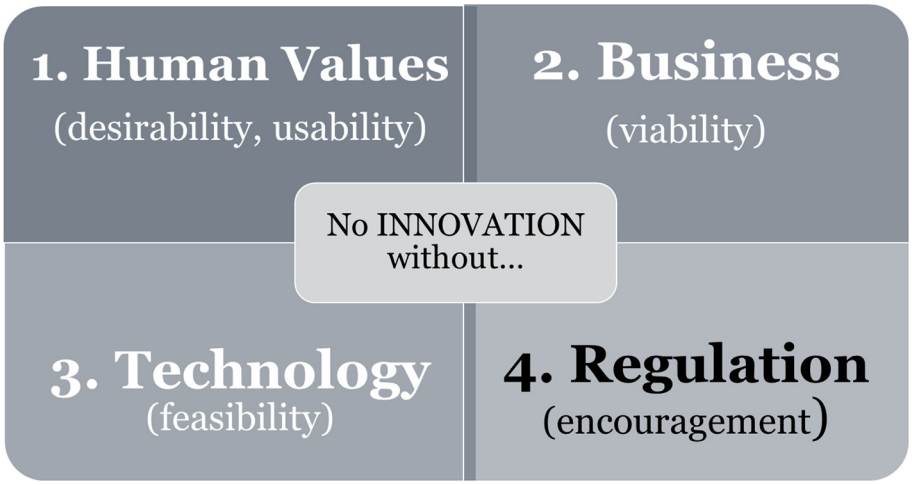

FIGURE 1 | The "four-leaf clover" of innovation. The model is derived from the Theory of Culture Development (altered from Figure 4 of von Thienen et al., 2019). Innovation depends on the requirement of all four leaf branches. Three of them (white letters) evolve directly from [human] culture. Novel needs [including environmental protection goals] based on human values (1) which are only viable if [economic] business (2) is possible in combination with the development of novel [technology] designs (3). As an indirect driver, encouragement by regulation (4) completes the successful leaf development.

international agreements such as the Cartagena Protocol under the United Nations Convention on Biological Diversity (CBD)[1].

Regulation of GMOs generally requires an Environmental Risk Assessment to be conducted before a GMO can be released into the environment. The Environmental Risk Assessment starts with the development of risk scenarios—hypotheses of what harm the GMO could cause to people or the environment. The type of data that needs to be collected prior to the release would be informed by these scenarios. Development of the hypotheses generally requires input and advice from a range of different scientific disciplines (see section Specific ERA Challenges Associated With Gene Drive Organisms of this paper).

KEY ELEMENTS TO REGULATION

Most regulatory systems aim to protect human and animal health and the environment while at the same time enabling research and development of beneficial products by modern biotechnology. Since there is no activity in life that does not carry some risk, both regulatory precaution and innovation principles need to consider risks caused by taking action or no action. Avoiding innovation, e.g., by overcautious and restrictive GMO regulations, might also increase the risk of biodiversity loss, food insecurity, and socioeconomic disasters in a time where human population growth, biodiversity loss, climate change, and decreasing natural resources are substantially impacting Earth (Bartsch, 2017). In this respect, evidence based decisions support more sustainable solutions. Decision makers must pay thorough attention to factors relating to both the production and use of such evidence (Redford et al., 2019).

Whilst most regulatory systems for GMOs have the same primary goals different regulatory systems in various jurisdictions may incorporate other issues. For example, Directive 2001/18/EC (EC—European Commission, 2009) recognizes the respect for ethical principles in the EU, and Member States may take into consideration ethical aspects when GMOs are deliberately released. Socioeconomic advantages and disadvantages of each category of GMOs authorized are considered in a report to be issued every 3 years by the EU Commission. The decision maker in the EU is—theoretically—not restrained from considering benefits. In Australia, decisions under the *Gene Technology Act 2000* cannot consider ethics and economics. However, the Australian States and Territories can introduce Policy Principles to consider these.

The Role of Regulation for Innovation

Regulation is important for framing innovation, since promotions and restrictions are vital factors guiding which products make it through the research and development stage. Political and economic contexts are important factors influencing technological development and the range of economic profit, and societal need determines technological priorities (see Chapter 6.3 of Redford et al., 2019). Innovation is only possible if new ideas match the desirability/usability for human values, viability of business, technological feasibility, and regulatory encouragement (see **Figure 1**).

[1]The Cartagena Protocol on Biosafety to the Convention on Biological Diversity defines a so-called living modified organism (LMO) in Article 3 (g) as "any living organism that possesses a novel combination of genetic material obtained through the use of modern biotechnology." Modern biotechnology is further defined in Article 3 (i) as "the application of: a. *In vitro* nucleic acid techniques, including recombinant deoxyribonucleic acid (DNA) and direct injection of nucleic acid into cells or organelles, or b. Fusion of cells beyond the taxonomic family, that overcome natural physiological reproductive or recombination barriers and that are not techniques used in traditional breeding and selection."

The technological feasibility of gene drives has developed very rapidly and research projects have been initiated across vector control and agriculture (National Academies of Sciences Engineering Medicine, 2016). It is too early to determine the success, business viability or public acceptability in many of these areas yet.

Human values find their expression in protection goals when it comes to the assessment of invasive species. Whether control actions are acceptable depends on the trade-off between environmental/health damage caused by the invasive species/pest/disease vs. undesired off-target effects of the controlling technology. The public concerns about GMOs will also influence this debate (Cormick and Mercer, 2017; Delborne et al., 2019; Hartley et al., 2019).

Environmental Protection Goals

Since supporting human and valued animal health is a universally accepted protection goal, this section will focus on the environment. The goal of environmental protection is avoiding harm and/or remediation of damage. Bartz et al. (2009) defined environmental damage as:

1) "A significant relevant adverse effect on a biotic or abiotic conservation resource
2) that has an impact on conservation

   a) values,
   b) ecosystem component, or
   c) its sustainable use."

This definition covers the purposes of conservation as defined in the CBD: "to protect conservation resources themselves and in their role as a part of ecological structures and functions and to safeguard their potential sustainable use."

The definition has three normative conditions, basically due to legal enforcement options: Only

(i) concrete (measurable) adverse effects,
(ii) adverse effects that lead to a decrease in "the value," and
(iii) adverse effects that are significant [environmentally relevant] can be damages.

The magnitude of adverse effects caused by invasive species is reviewed and classified in more detail by Blackburn et al. (2014). However, it is important to re-iterate that regulatory decision making should take into account the ecological consequences of applying/not applying control measures (including GD) when it comes to remediation of damage. A well-designed risk assessment helps to manage the tension between a desire for caution regarding the risk of intervention and worry about the risks of non-intervention (Wareham and Nardini, 2015). A historic example of intervention for the beneficial eradication of a disease is malaria in Europe (**Box 2**).

The GD organism released for invasive species control is—although "invasive" to some extent by definition—a beneficial species since it mitigates damage to the environment, human economy or human health. However, the beneficial organism should not turn into an undesired invasive species.

BOX 2 | Comparative assessment for protection goals—Eradication of malaria in Europe:

Malaria was a widespread disease in several parts of Europe including Germany (Dalitz, 2005). The eradication was achieved with the help of chemical and sanitary measures to kill the mosquitoes which transmitted malaria (De Zulueta, 1998; WHO, 2016). These included drainage of wetlands in the Oderbruch west of Berlin in the eighteenth century and broad-spectrum pesticide sprays in the Italian Po-Valley. It is not known what unintended environmental damages occurred from these interventions.

Malaria could re-establish by re-introduction of mosquitoes via global trade shipments or tourists arriving from infested countries, combined with the possibility of the receiving environment in the EU being permissible due to global climate change conditions (Schröder and Schmidt, 2014). It is conceivable that GD may become an option to target malaria via eradication or substitution of vector insect populations.

International Legal Frameworks

International instruments provide valuable frameworks for the regulation of GD (**Table 1**).

Since GD applications are intended to release organisms that become established in the environment and may spread across landscapes, countries have a responsibility for transboundary risk assessment and liability of damage caused by such releases. Many—but not all—countries work under the umbrella of the Cartagena Protocol on risk assessment, information exchange, and further harmonized regulation of transboundary movements of GMOs (Tung, 2014). It is likely that regional and bilateral approaches will be established first before harmonization can be expected at higher international levels.

There is an international customary rule that a country must prevent and provide compensation for damage wrongfully caused from its territory to other states (see more details in Redford et al., 2019). The International Law Commission of the United Nations has published draft articles on the responsibility of countries for internationally wrongful acts. These provide an obligation to make reparation for "any damage, whether material, or moral, caused by the internationally wrongful act of a State" (United Nations, 2001). Whether these rules may apply for negative effects caused by GD releases is—as far as the authors know—not completely solved yet.

The EU Regulatory System

The EU has elaborate guidelines on application of the precautionary principle which include a preliminary evaluation of risks and uncertainties to determine when the principle is triggered (EU, 2000). The precautionary principle has been taken into account in the drafting of the two statutory regimes:

- Contained use (Directive 2009/41/EC for microorganisms EC—European Commission, 2009, in various EU Member State regulations also for other organisms)
- Release into the environment [Directive 2001/18/EC (EC—European Commission, 2001)].

Contained use in the laboratory is the first step in developing safe and sustainable GD applications. There is currently an initiative

TABLE 1 | International legal frameworks (adapted with permission from Redford et al., 2019).

Instrument	Description	Relevance for gene drive
Convention on Biological Diversity (CBD) Adopted: 1992, Entered into force: 1993 Parties: 196	Global legal framework addressing conservation, sustainable use and sharing of benefits of biodiversity	Creates obligations for each Party to manage risks associated with living modified organisms that could have a negative impact on biological diversity [art. 8(g)] and framework for access and benefit sharing relating to genetic resources (art. 15).
Cartagena Protocol on Biosafety to the Convention on Biological Diversity (Cartagena Protocol) Adopted: 2000, Entered into force: 2003 Parties: 171	Protocol to CBD intended to ensure the "safe transfer, handling and use of living modified organisms resulting from modern biotechnology that may have adverse effects on biological diversity..." (art. 1)	Requires sharing of risk related information between exporting and importing Parties and provides guidelines on methodology for environmental risk assessments and considerations in decision-making.
Nagoya-Kuala Lumpur Supplementary Protocol on Liability and Redress to the Cartagena Protocol on Biosafety (Supplementary Protocol) Adopted: 2010, Entered into force: 2018 Parties: 42	Supplementary Protocol to Cartagena Protocol intended to provide rules and procedures for liability and redress relating to living modified organisms	Provides for national frameworks requiring response measures and assigning civil liability in event of damage resulting from living modified organisms which find their origin in transboundary movement.
Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization to the Convention on Biological Diversity (Nagoya Protocol) Adopted: 2010, Entered into force/not entered into force	Protocol to CBD providing international framework for access to genetic resources and sharing of benefits arising from their utilization	Applies to genetic resources that serve as source material for synthetic biology research. Creates ABS framework based on traceability and transfer of material.

for EU wide harmonization on risk assessment and authorization for such use (van der Vlugt et al., 2018) since responsibility falls to the authorities of EU Member states.

The Directive 2001/18/EC (EC—European Commission, 2001) sets out a step-by-step approach for introduction of a GMO into the environment, with evaluation of impacts on human health and the environment. Information is required about parental / donor / GM organism, and the receiving environment. Risk assessment follows a case by case and step by step approach (see **Figure 2**). It is important to identify the characteristics which may cause adverse effects, e.g., effects on the dynamics of populations of species in the receiving environment and the genetic diversity of each of these populations.

Regulation of Gene Drives in the EU

For GD to control invasive species, the (intended) effect on targeted (invasive) species is not regarded as adverse but beneficial since the invasive species already negatively affects other species in the receiving environment. The environmental risk assessment follows in detail the Guidance Documents published by the European Food Safety Authority (EFSA). Since GD is likely to be first applied in form of GM animals, the structure of the EFSA GD document on ERA of GM animals is shown in **Figure 3**.

The Australian Regulatory System

Regulation of GMOs

Australia has specific legislation to regulate activities with GMOs to protect people and the environment. The *Gene Technology Act 2000* (Commonwealth of Australia, 2000) and the Gene Technology Regulations 2001 (Commonwealth of Australia, 2001), covers activities with all GMOs, including microorganisms, plants and animals, both in contained facilities and when released into the environment.

The objective of the legislation is to "protect the health and safety of people, and to protect the environment, by identifying risks posed by or as a result of gene technology, and by managing those risks through regulating certain dealings with GMOs."

Regulation of contained GMOs typically focuses on the suitability of containment. For a GMO intended to be released into the environment, protection of the environment is typically achieved by following a step-wise development process (OECD, 1986): data from initial contained research, overseas release/s or release of a similar GMO inform authorizations for small, short term, confined trials where the GMO is removed from the environment once the trial is finished. Each application for release into the environment requires a case-by-case risk assessment and tailored risk management plans, combined with mandatory consultation requirements, including formal consultation with the Australian Minister for the Environment.

The Risk Analysis Framework (OGTR, 2013) explains the Regulator's methods for risk analysis. It mandates a comparative, problem formulation approach where risk scenarios are used to develop credible causal pathways whereby a GMO may cause harm to people or the environment.

Regulation of Gene Drives in Australia

Recent amendments to the GT legislation[2] provide clarity on the regulatory status of organisms developed using a range of new technologies. Work with organisms containing a functional engineered gene drive will require a specific case-by-case evaluation of the risks and specific risk management of activities with these organisms. This assessment permits information gathering as well as monitoring of the progress of research in

[2]These amendments, arising from the Gene Technology Regulator's Technical Review of the Regulations (http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/reviewregulations-1), come into force 8 October 2019.

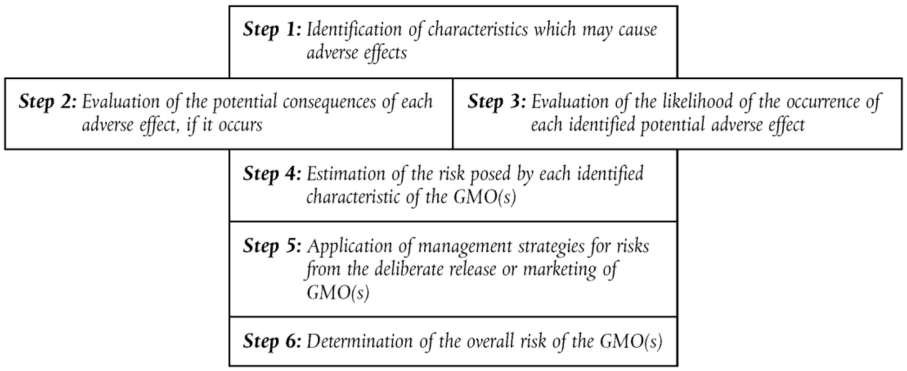

FIGURE 2 | The six steps in the analysis of Environmental Risk Assessment (ERA) in the European Union according to Directive 2001/18/EC (EC—European Commission, 2001).

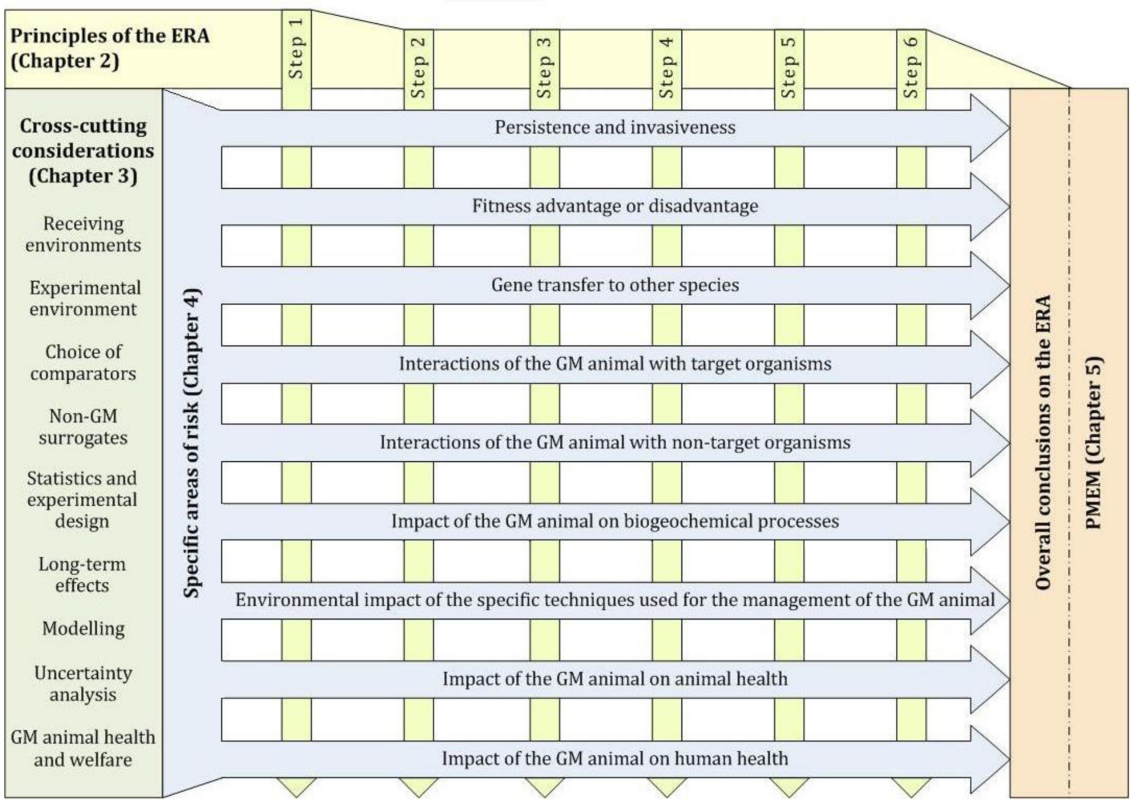

FIGURE 3 | Structure of the EFSA Guidance document on ERA of GM animals, which would apply to GD insects. In late 2018, EFSA received a new mandate from the EU Commission on GMOs engineered with gene drives (gene drive modified organisms) and their implications for RA methodologies. EFSA is requested to identify potential risks in terms of impact on human and animal health and the environment. EFSA is also asked to identify potential novel hazards and to determine whether the existing guidelines are adequate or whether there is a need for updated guidance. EFSA is not requested to develop guidelines for the RA of gene drive modified organisms. Thus, the current guidance on the ERA of GM plants and animals are still valid (EFSA Panel on Genetically Modified Organisms., 2010, 2013).

this new area. Case-by-case evaluation will take into account any risk-mitigating approaches such as molecular (e.g., split drives, daisy drives, and synthetic targets whereby the gene drive is engineered to prevent uncontrolled spread), environmental or physical containment.

The 2017 legislation review (Commonwealth Department of Health, 2018) observed that "There is an identified need to determine the most appropriate approach for regulating the environmental release of genetically modified gene drive organisms (as well as any additional requirements for contained

work)." This may lead to future consideration of whether changes to policy are needed to address issues raised by GD GMOs, particularly in the context of intentional environmental releases.

SPECIFIC ERA CHALLENGES ASSOCIATED WITH GENE DRIVE ORGANISMS

Any ERA should start with the Problem Formulation step in which the ERA scope is determined, including the protection goals and the risk hypotheses. The nature of the GD and its ability to spread could lead to jumping over gradual introduction steps (laboratory—small scale release—large scale release) into the environment. Careful consideration of data gaps related to this "short-cut" is inherently important.

Gene drives can be designed to be self-limiting, whereby the drive will only work for a limited number of generations or is limited spatially. If a GD is designed to be self-limiting, the evaluation of population suppression GDs needs to consider the limited GD persistence in the environment and the required efficacy of the GMO release.

If a GD is designed to be self-sustaining, population suppression GDs need to consider the higher persistence in the environment and the smaller number of required releases of modified organisms. The ecological consequences of extinction also need to be considered. In the case of population replacement and substitution GDs, assessors have to place a greater emphasis on the exact heritable trait compared to GDs that cause removal of the organism from the environment.

One crucial aspect of GD is the cargo—the genetic elements that will be dispersed through the population. The recent case of hybridization and introgression of genetic elements from a released transgenic mosquito strain in Brazil (Evans et al., 2019) points to the key elements of ERA: What is the harm and how likely is this to occur? This particular case did not involve a gene drive, but it illustrates a point that would apply to gene drives. The genetic elements that were introgressed were from the transgenic mosquito genetic background, rather than an introduced transgene. Therefore, any particular effect that might be observed cannot be attributed to genetic engineering. This is an important paradigm for the internationally agreed comparative ERA approach.

A gene drive which is designed to kill an organism after reproduction would have a different likelihood to cause harm than one which prevents the organism transmitting disease.

In the past few years GD has been subject to regulatory consideration in the US[3], Australia[4] in Europe (BVL as office for the German Biosafety Commission "ZKBS"[5] and by researchers (Oye et al., 2014; Akbari et al., 2015) in contained use facilities. To date, no government decisions on gene drive have been made for environmental releases. Nevertheless, the question of whether new environmental risk assessment (ERA) challenges are associated with GD organisms has also been addressed by scientific organizations (Redford et al., 2019) and researchers (Esvelt et al., 2014; Collins, 2018; Rode et al., 2019).

ERA utilizes a reasoned, structured approach to address uncertainty based on scientific and technical evidence (Wolt et al., 2010). Release of GD organisms into the environment currently has a high degree of uncertainty about how they would behave. Whilst modeling can help predict the outcomes (de Jong, 2017), additional data is required to determine if harm could occur during these kinds of releases. This additional information to improve risk assessment is data which is critical to assess risk to the environment (e.g., data on altered phenotype and population data rather than molecular data) (Layton et al., 2015).

Guidance on how to identify significant risks from organisms can be obtained from our experience with those organisms that cause harm. For example, there is a wealth of information on plants (including crops) that cause harm. These plants are generally known as weeds and weed scientists have well-developed methods to assess risks and harms from weeds (Pheloung, 2001; Standards Australia, 2006; Bourgeois et al., 2019). These methods have been successfully modified for use in the risk assessment of GM crops (Keese et al., 2014).

Ellstrand (2018) reviewed 14 well-documented situations where GMOs have been detected in wild or feral plant populations. These have occurred due to seed or pollen movement. Using the core principle of regulatory risk assessment "exposure" x "hazard" = "risk, gene flow (including GD) is the "exposure" component of the equation." Despite gene flow occurring, to date an environmental "hazard" became apparent only in very few of the studied cases. The most significant of these is glyphosate-tolerant creeping bentgrass which has become a significant weed of irrigation canals in Oregon, USA (National Research Council, 2017). This weed can be controlled using other herbicides, but these chemicals may be less desirable, particularly near waterways (Beckie et al., 2004).

Similarly, for animals there is guidance on what harms pest animals cause in different environments from risk assessors who currently control pest animals (e.g., SA pest animal risk assessment guide). There is also guidance and vast experience from release of biocontrol agents to control invasive pests or pathogens in many countries of the world (Saunders et al., 2010; McColl et al., 2014), which would be applicable to gene drives.

Currently most GMOs are applied in the agricultural sector. GDs are different as most of the proposed applications are intended to modify wild populations. There are some proposed applications in plants (Neve, 2018), but generally. GD applications are seen as less relevant in plants or for use in agricultural systems (Duensing et al., 2018).

GD in wild animals providing fitness advantages for the hosting individuals will undoubtedly increase the environmental exposure of a GMO. It is thus very important to generate reliable data in the laboratory and from contained releases (e.g., islands) before the introduction into borderless/expansive

[3]https://www.aphis.usda.gov/aphis/ourfocus/biotechnology/am-i-regulated/Regulated_Article_Letters_of_Inquiry

[4]http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/53139D205A98A3B3CA257D4F00811F97/$File/Guidance%20on%20gene%20drives.pdf (accessed January 02, 2020)

[5]http://www.zkbs-online.de/ZKBS/EN/03_Fokusthemen/Gene%20Drive%20Systems/Gene%20Drive%20Systems_node.html (accessed December 30, 2019)

environments. Nuclease-based GDs are the most advanced and thus the major focus: According to Redford et al. (2019) three types of information about the target and non-target species are required before implementing a gene drive strategy:

"– *Genetic and technical information needed include how to breed and conduct controlled experiments in the target species. Gene drive research also requires the availability of genome editing technology in the focal species or a related species, and the availability of an annotated reference genome to identify potential targets and design gRNAs that are specific of these loci (Moro et al., 2018).*

– *Ecological and evolutionary data on potential non-target species includes quantification of gene flow between target and non-target species (hybridization or horizontal gene transfer), checking for the presence of potential target sites in non-target species, and appropriate modeling of food web structure to forecast long-term ecosystem impacts (Moro et al., 2018).*

– *Ecological information needed includes behavioral and demographic data (e.g. spatio-temporal variation in size; Moro et al., 2018), and a good understanding of the mating system and of gene flow between populations (e.g. quantifying dispersal ability as well as anthropogenic dispersal; Webber et al., 2015). Spatially explicit theoretical models can help predict gene drive dynamics."*

Two types of modeling are available supporting the ERA in the (inherent) light of uncertainty: population genetic models (e.g., de Jong, 2017) and spatial population models (e.g., Sánchez et al., 2019)

A threat for the sustainable use of GD systems is the development of resistance in target species. Prominent examples have already been identified in laboratory experiments (KaramiNejadRanjbar et al., 2018). Improved molecular designs may counteract rapid resistance evolution (Champer et al., 2019).

Whatever detailed guidance for GD will be developed in the future, it is important to take the lessons learned from the Cartagena Protocol Guidance on Risk Assessment of LMOs into account (Hokanson, 2019).

DECISION MAKING

Debates regarding the regulatory status of GD organisms generally follow a comparative approach with GMOs and with conventional organisms obtained by mutation or breeding. However, in case of GD organisms the likelihood and spread of genes into target and non-target populations is increased relative to comparators. Thus, it is the consequence of—successful—gene drive applications that needs to be finally assessed by decision makers.

As Redford et al. (2019) pointed out, *seeking to reduce epistemic uncertainty by performing a risk assessment on emerging technologies may require research activities that themselves pose some risk*. There will be tradeoffs between reducing uncertainty and avoiding risk that challenge the decision making process. Conducting field trials on isolated islands first and/or molecular confinement measures may be suitable steps forward.

Risk assessment and decision making for classical biological control for invasive species was reviewed by Teem et al. (in preparation), providing considerations involved in releasing an organism into the environment. The use of natural enemy species as biological control has been widely used and is accepted as an environmentally sound and effective means of reducing or mitigating the effects of pest species. Such natural enemies species have been successfully used to control invasive species all over the world.

Regulators and policymakers need to become familiar with the technical aspects of GD as well as the societal impacts. Of particular importance is public participation, stakeholder involvement and capacity building in "Release States." Regulators need to contribute and encourage open and trustworthy GD research. A critical review of such "responsible" GD introduction is provided by Kuzma (2019) who argues that "external experts, stakeholders, and citizens with specialized and local knowledge" should be consulted in a more transparent way (Kuzma, 2019). This need for a change in communication style was also discussed by Brossard et al. (2019). Potential examples of how stakeholders are involved in specific projects can be found at the Target Malaria Project[6].

HARMONIZATION OF REGULATION

In order to facilitate free global trade, internationally harmonized regulations are needed. However, it is not clear for GD whether this will be possible. It would require an international consensus for regulation which would require an organization to able to advance and coordinate this harmonization process. The questions around what is possible and who might advance this harmonization process will certainly be on the agenda of international conferences, e.g., under WHO or FAO leadership. One of the most crucial points here is the need for risk/benefit analysis in order to increase public awareness and also the awareness of the regulatory authorities and policy makers.

PREPARING FOR FUTURE GENE DRIVE APPLICATIONS

Risk and regulatory considerations for gene drive organisms will evolve considering the speed of introduction into the environment and the geographical location (Harvey-Samuel et al., 2019). At a workshop held at the Lorentz Center in Leiden 2017[7], participants gave a rough forecast for the next 10 years:

Timeline of potential first environmental release of GD organisms:

- *Mus musculus* 2023
- *Anopheles gambiae 2026*
- *Felis catus* 2028
- *Rhinella marina* 2030.

[6]https://targetmalaria.org/

[7]https://www.lorentzcenter.nl/lc/web/2017/872/info.php3?wsid=872&venue=Oort, accessed July 30, 2009.

CONCLUSION

The increase in efficacy and decreasing costs will revolutionize the tools that science-driven economies will apply to fight against invasive species. These will include modern tools like GD. The right balance between precaution and innovation needs to be found for the benefit of society. The policy around regulation needs to balance the public's need for health, food, feed, and environmental safety with the economic costs for developers, growers, shippers and processers without wasting or damaging environmental resources. The importance of a globally harmonized regulatory approach is key to successful innovation. There is a general agreement that GD is a very powerful tool that needs careful and thorough evaluation before any release into the environment should be granted. It is still unclear whether a self-limiting GD is likely to be favored by regulators for approval compared to self-sustaining GD. Risk assessments for all gene drives will be on a case by case basis, so it is difficult to predict how different GD will be evaluated by risk assessors before they are assessed by regulators. Gene drive mouse and mosquitoes for invasive species control will be the likely test case for public acceptance of gene drive technology. A broad range of expertise, including ecologists, conservation geneticists, and nature reserve managers need to be involved. Responsible policy making benefits from engagement with stakeholders, policymakers, and local communities (Sirinathsinghji, 2019).

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Familiarity in the Context of Risk Assessment of Transgenic Crops: Focus on Some Countries in the Americas

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Problem formulation is the formal opening stage of a risk assessment that determines its purpose and scope and hence guides the gathering of information data. The concepts of familiarity and history of safe use are an integral part of problem formulation. These concepts do not replace the case-by-case approach and are not taken as safety standards but are valuable components of the process that shape the generation of plausible, testable risk hypotheses. The International Life Sciences Institutes in Brazil and Argentina have facilitated numerous discussions on the scientific principles for risk assessment of transgenic crops in the Latin American region in the past 5–6 years. The session held at ISBR 15th elaborated on the familiarity concept and derived tools and their role in the evolution of risk evaluation criteria. Examples of how different countries in the Americas interpret and apply these conceptual tools show that familiarity is a valuable concept, although terms are very often confused and vaguely defined. Formalizing these terms with clear definitions and scope of application in guidelines and regulatory documents would reduce ambiguity, enhance predictability, and add transparency to the evaluation processes.

Keywords: familiarity, history of safe use, risk assessment, problem formulation, regulatory framework, harmonization

INTRODUCTION

Risk assessment criteria for transgenic organisms have been set decades ago and are still current, built on the following: case-by-case, comparative assessment, tiered approach, and consideration of the weight of evidence. However, as science moves forward, new developments and knowledge make it necessary to periodically update and/or adjust these criteria (Borges et al., 2018).

Problem formulation has been defined as the "formal, structured, opening stage" of the risk assessment (Patton, 1998). It was originally described in the Environmental Protection Agency's Framework Report (Norton et al., 1992; EPA, 2014) as a conceptual model that considers the values to be protected, the data needed, and the analyses to be used. Problem formulation determines the risk assessment purpose and scope, guiding the gathering of information and data. It presumes the formulation of risk hypotheses, which in turn are shaped by previous experience and knowledge and will be tested against available data (Wolt et al., 2010).

The concepts of familiarity and history of safe use (HOSU) are an integral part of problem formulation, as the availability of existing information is a critical element that adds to the weight of evidence. The Organisation for Economic Co-operation and Development (OECD) was among the first to articulate some of the core principles of familiarity for environmental risk assessment of genetic modified organisms (GMOs) in the Blue Book, back in 1986 (OECD, 1986); and later (OECD, 1993), the basic principles for environmental risk assessment were consolidated and globally accepted to this day. Regarding the food and feed safety assessment of GMOs, the Codex Alimentarius issued specific principles a decade later that constitute the global standard reference (Codex Alimentarius, 2003).

The concept of familiarity involves knowledge and experience that can be used for risk analysis and helps to identify if and what additional knowledge is really needed; therefore, it is not equivalent to safety (Constable et al., 2007).

In September 2018, a workshop facilitated by the International Life Sciences Institutes in Argentina and Brazil discussed the practice of the risk assessment of GMOs in Latin America and identified that the terms "familiarity" and "HOSU" were not clearly defined or were not consolidated as a concept in the literature or guidelines. This group concluded that a consensus would be required on these terms as important tools with harmonization potential for regulatory criteria (ILSI Brasil, 2018). The interpretation and practical implications of the use of these terms by risk assessors in other countries of the Americas were recognized as very relevant. The United States Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) guideline for "extensions" (USDA-APHIS, 2016), as well as the Canadian approach to similar plants with novel traits (PNTs) (Canada/CFIA, 2018) are excellent examples of how experience with risk assessment and accumulated knowledge can be leveraged to enhance efficiency while keeping a high regulatory standard.

A CAST publication from the same year (Council for Agricultural Science and Technology [CAST], 2018) also addressed familiarity as a key element to reduce the time and effort for decision making and be more efficient in the use of public resources. The following quote gives the flavor of the discussion: "Regulatory agencies. . .should be prepared to focus questions on identifying new pathways to risk assessment endpoints associated with products that are **unfamiliar** and that require more complex risk assessments."

Based on these precedents, ILSI Argentina and Brazil held a session at ISBR 15th (April 2019) to elaborate on the familiarity concept and derived tools, and their role in the evolution of risk evaluation criteria. Examples of how different countries in the Americas interpret and apply these conceptual tools were discussed and are presented here.

FAMILIARITY IN THE CONTEXT OF PROBLEM FORMULATION

Data to support problem formulation can be derived from multiple sources; for the case of transgenic crops, published literature on the biology of the crop, genes, and expression products, and existing documentation on molecular, compositional, and agronomic/phenotypic data are all relevant sources. This information is often available and can help refine and/or reduce the hypotheses that need to be tested for risk characterization (Garcia-Alonso, 2010). In this way, the study plan will include only those tests that must be conducted, as indicated by the problem formulation exercise (Romeis et al., 2009).

By definition, "familiarity" (knowledge and experience) helps in addressing uncertainty in the risk assessment because it is based on preexisting knowledge, experimental evidence, and experience gained over time (OECD, 1993; Hokanson et al., 1999).

Three main knowledge-based factors have driven the evolution of risk assessment criteria for transgenic crops in many parts of the world during the last decade, namely, advances in the knowledge of the intrinsic plasticity of plant genomes (Doebley et al., 2006; Weber et al., 2012; Anderson et al., 2016), of the genomic/genetic effects of transgenesis compared to conventional breeding (Baudo et al., 2006; Batista et al., 2008), and of the natural variability of biochemical composition of the most important crop plants (OECD, 2006, 2002-2012; Ricroch, 2012; Venkatesh et al., 2014; CERA, 2015). This body of knowledge, along with extensive data from the characterization of transgenic events, plus the experience of use of transgenesis in plant breeding, has greatly increased the level of familiarity with the technology (Burachik, 2010; Schnell et al., 2014; Beker et al., 2016).

Experience with the practice of risk assessment is also in itself a substantial component of familiarity, as experienced risk assessors will integrate scientific advances to their own risk assessment experience, contributing to the evolution of evidence-based criteria (USDA-APHIS, 2018).

As for the term "HOSU," a high level of ambiguity can be found in the language used in guidelines or international documents. According to the OECD, "A long HOSU is a reassuring and practical starting point, for evaluating the safety of a novel food" (OECD, 1999), although "long" is not defined. Similarly, vague language is found in regulatory guidelines: "A substance may be considered to have a HOSU as a food if it has been an ongoing part of the diet for a number of generations in a large, genetically diverse human population where it has been used in ways and at levels that are similar to those expected or intended in Canada" (Health Canada, 2006), or "related, among others, with consumption habits and the massive consumption of the GMO in other countries over years" (Ministerio de Agricultura Ganadería y Pesca, 2013). Specific dates are also found as defining HOSU: Europe defines novel foods as "any food that was not used for human consumption to a significant degree within the Union before 15 May 1997," or, for traditional foods from third countries, "foods should have been consumed in at least one third country for at least 25 years as a part of the customary diet of a significant number of people" (Engel et al., 2011; EU, 2015).

Although HOSU and familiarity are related concepts, these are not synonymous, even when these terms are frequently used interchangeably. HOSU should be preferably used for traditional

uses, of which scientific procedures or formal knowledge would not necessarily be available or may be limited. Familiarity, on the other hand, refers to the body of knowledge (evidence/data) and experience (of use, but also with risk assessment) with technologies and products that have undergone a risk assessment process or for which substantial data are available (**Figure 1**). Ambiguous language can create confusion and ultimately leads to discretionary interpretations and less predictable risk assessment processes (Wasmer, 2019). To exemplify the relevance of clear definitions, in the specific case of transgenic comparators for field studies, using HOSU as an acceptance criterion would be discretional. Familiarity, on the other hand, would describe the availability of documented knowledge that would allow for using these, as well as null segregants as suitable comparators.

Clear and consistent definitions enhance transparency and facilitate conceptual harmonization for modern, evidence-based risk assessments.

THE USE OF FAMILIARITY AND A HISTORY OF SAFE USE IN THE DECISIONS OF THE BRAZILIAN NATIONAL BIOSAFETY TECHNICAL COMMISSION

The current GMO legislation in Brazil centers around the Biosafety Law and Decree (BRASIL, 2005), and Norms and Technical decisions[1] issued by the National Biosafety Technical Commission (CTNBio).

The heart of the Brazilian GMO Biosafety policy is CTNBio[2], a consulting and deliberating multidisciplinary collegiate body that formulates the norms, examines the evidence, and authorizes any activity related to GMOs.

Even though neither the Biosafety Law and Decree nor CTNBio's Normative Resolutions mention the terms "familiarity" or "HOSU" in the context of risk assessment of GMOs and their by-products, these concepts are implicit in the assessments performed by its members. As experienced scientists (all members of CTNBio must hold a doctorate degree, have acknowledged technical competence, and should have been professionally active in the biosafety, biotechnology, biology, human or animal health areas, and the environment), the use of the scientific method is an intrinsic part of their analysis.

In fact, observing the many review processes held for the commercial release of GMOs in the last 20 years[3], we note the use of the terms "history of use," "safe use," "safe consumption," "safe history," and "HOSU" in several documents. However, the term "familiarity" is not used. We believe that this is due to a lack of a standard definition and therefore of a misconception of the term. CTNBio's risk assessors, in writing their technical opinions, infer that there is knowledge (evidence/data) and experience in the use of technologies and products, in particular, those who have undergone a risk assessment process or for which substantial data are available, in other words, familiarity.

The Brazilian Biosafety Law establishes that all activities related to GMOs in the country must be "guided by the drive for attaining scientific development in the biosafety and biotechnology area, the protection of life and human beings, of animal and plant health, and the compliance with the principle of environmental precaution." In addition, it is the responsibility of the proponent to establish that the proposed activity will not (or is very unlikely to) result in significant harm.

Normative Resolution No. 05 of CTNBio (BRASIL, 2007) mentions "the history of use for food and feed of the GMO unmodified parent" and "the history of cultivation and usage of the GMO unmodified parent in the environment" as key pieces of information to consider. In addition, CTNBio Technical Decisions have consistently reflected (even with no mention in the law) the application of conceptual tools based on familiarity, as data for human and animal health risk assessment performed in other parts of the world are considered. However, as established in the same normative, environmental evidence for risk assessment has to be generated in the ecosystems in which the particular plant will be cultivated.

National Biosafety Technical Commission has evaluated and approved four yeast strains for the production of first- and second-generation ethanol, three yeast strains, and seven microalgae for oil production, in addition to a large quantity of animal vaccines, until 2018. Recently, four varieties of GM corn have been approved for marketing exclusively for human and animal consumption, although they cannot be grown in Brazil because they have not been tested in the Brazilian edaphoclimatic conditions as required; however, these assessments did consider available information generated elsewhere, and therefore, the concept of familiarity was used.

Finally, as stated in the Brazilian Biosafety Law: "CTNBio shall monitor the development and technical-scientific progress attained by the biosafety, biotechnology, bioethics and related areas, with aims at increasing their capacity of protecting human, animal and plant health and the environment." This provision legally ensures that CTNBio's decisions are based on the most current scientific knowledge and state of the art.

THE REGULATION OF AGRICULTURAL BIOTECHNOLOGY AND SCIENCE: A CANADIAN PERSPECTIVE ON THE CONCEPTUAL TOOLS FOR PROBLEM FORMULATION

During the scientific consultations carried out in the late 1980s on biotechnology-derived plants, it was agreed that the regulatory scope should be focused on plants with traits sufficiently different from those already present in the species, as to require a risk assessment. This led to the recommendation that the product and not the process would be regulated, and the

[1] Available at: http://ctnbio.mcti.gov.br/resolucoes-normativas/

[2] Available at: http://ctnbio.mcti.gov.br/inicio

[3] Available at: http://ctnbio.mcti.gov.br/en/liberacao-comercial#/liberacao-comercial/consultar-processo

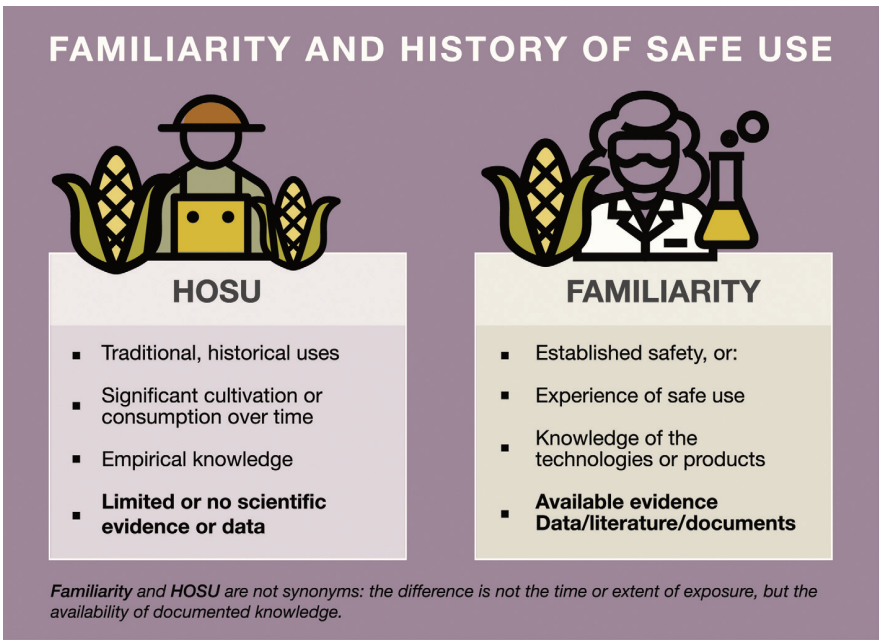

FIGURE 1 | Proposed differences between HOSU and Familiarity.

scientific perspective that came from these consultations was that plants derived through genetic engineering were not necessarily any riskier than those derived through chemical mutagenesis or other breeding techniques. This resulted in a regulatory approach that created the basis for the effective incorporation of science into policy, giving rise to the articulation of the 1993 Federal Regulatory Framework for Biotechnology[4]. This framework described an approach to biotechnology, based on the use of science-based safety assessments and risk management, aimed at protecting human and animal health, and the environment and at the same time providing an environment that allowed for innovation. All current regulatory frameworks for transgenic plants incorporate the need for a risk assessment prior to environmental release, to identify and evaluate the risks associated with the release and cultivation of these plants using a comparative approach.

Key to the environmental risk assessment is a thorough knowledge of the crop species that has been subject to modification by biotechnology to express a new trait. This knowledge is fundamental to conducting a comparative risk assessment. The concept of familiarity is used to identify and evaluate environmental risks that may be associated with the release of a transgenic plant and to inform management practices that may be needed to mitigate recognized risks. In Canada, this requirement is satisfied by the creation of individual-crop biology documents. These documents describe the behavior of the crop species specifically in the Canadian environment and include a description of relevant parameters (plant growth, reproduction, interactions with related and unrelated species, management practices, etc.) to inform the risk assessment (CFIA, 2017). Although similar in focus to the consensus documents developed by the OECD (2006), the Canadian document describes management conditions and environmental interactions for the unmodified species that are specific to the Canadian environment, and uses the familiarity with the cultivation and management of a species as the basis to identify potential hazards during the safety assessment.

Using familiarity as a guiding principle and considering pathways to harm, a hypothesis that growing a certain GM crop will cause no harm is really a hypothesis that growing the GM crop will cause no greater harm than that cultivation of the non-GM crop it may replace. For the risk assessment, "a hypothesis that growing a certain GM crop will pose no unacceptable risk is really a hypothesis that any increase in risk caused by growing the GM crop will be acceptable" (Raybould and Macdonald, 2018).

The principles of the comparative risk assessment, the use of familiarity, and the Canadian product-based approach (CFIA, 2017) were evident in a recent incident when Canadian regulators, like those in other countries, became aware that petunias that had been genetically engineered to produce orange flowers by using a gene from corn were potentially present in Canada. Regulators in Canada considered relevant information and scientific rationale, and determined that the GM petunias pose no more risk to the environment than conventional petunias, and in line with the product-based approach, they would not be regulated in Canada. Since there was no scientific evidence that the GM petunias posed any risk to the environment, distributors or producers of the GM petunias were not required to remove them from the supply chain.

[4]Available at: https://www.ourcommons.ca/Content/Archives/Committee/352/sust/reports/03_1996-11/chap2-e.html

For the crops we know well, the concept of familiarity and the comparative risk assessment approach has provided a useful paradigm for risk assessments. In fact, today most of the maize, soybeans, and canola grown by Canadian farmers are a product of biotechnology. As techniques such as gene editing push more new varieties forward to the marketplace, these sound principles for risk assessment, anchored in a strong policy framework, will allow Canadian farmers safe access to these new varieties.

CONCEPTUAL TOOLS BASED ON FAMILIARITY. TRANSPORTABILITY OF FIELD STUDIES FROM BRAZIL TO ARGENTINA: A CASE STUDY

The conceptual framework for data transportability (DT) builds on the premise that results from well-designed studies conducted for the environmental and food/feed risk assessment of transgenic crops may be relevant and therefore transportable to other geographies (Garcia-Alonso et al., 2013). This concept focuses not only on methodological quality but also on the familiarity with crops, traits, and receiving environments.

Bean crop (*Phaseolus vulgaris*) production took relevance in Argentina in the 70s as an alternative for rotation with other crops. One of the main diseases causing important yield losses is golden mosaic, caused by the *Bean Golden mosaic virus* (BGMV). In 2011, a transgenic bean resistant to BGMV was approved in Brazil for cultivation and consumption, developed by EMBRAPA (*Brazilian Agricultural Research Company*), through an RNA interference mechanism (BRASIL, 2011). ILSI Argentina's Biotechnology Working Group was interested in testing the applicability of the framework to a real case and, to this end, convened a subteam to discuss this particular case as an example.

A set of regulatory field studies carried out by EMBRAPA in Brazil were reviewed to discuss their transportability to the argentine receiving environment. This discussion considered that information generated in field trials is transportable, provided that trials are properly designed and conducted in diverse agroclimatic environments, allowing for the expression of any biologically relevant phenotypic differences. Under these considerations, sites selection, methodologies, and agronomic management of the studies were examined with focus on protocol and end point consistency, record keeping, and traceability. Familiarity with the crop and the bean cultivation zones in Argentina was also considered. The group concluded that the trials were transportable from Brazil to Argentina and might be eventually applicable to a risk evaluation process, provided that assessment end points would respond to the risk hypotheses identified according to regulatory requirements (Vesprini et al., 2019).

CONCLUSION

The concepts of familiarity and HOSU are an integral part of problem formulation. Although related concepts, they are not synonymous, in spite of the fact that they are often used interchangeably. Ambiguous language leads to discretionary interpretations and less predictable risk assessment processes. Clear and consistent definitions are needed to enhance transparency and facilitate conceptual harmonization for modern, evidence-based risk assessments.

This document intents to highlight the need for clearer definitions of these terms for the case of transgenic crops and propose to differentiate both terms based on the availability of documented knowledge. In this way, *Familiarity* should refer to the body of knowledge and experience with technologies and products that have undergone a risk assessment process or for which substantial data are available. *HOSU*, on the other hand, should be preferably used for traditional uses, of which scientific procedures or formal knowledge would not necessarily be available or may be limited.

The continued commitment in the practice of risk assessment of those who have direct responsibility for regulatory oversight leads to the integration of scientific advances in their own risk assessment experience, thus contributing to the evolution of evidence-based criteria. In other words, it allows for integrating familiarity into regulatory decisions. Collaboration among regulatory agencies is essential to this end.

AUTHOR CONTRIBUTIONS

All authors participated in the drafting of this manuscript as individual experts in their fields, and the authors are solely responsible for the contents. Any views expressed in this manuscript are the views of the authors and do not necessarily represent the views of any organization, institution, or government with which they are affiliated or employed.

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First Field Release of a Genetically Engineered, Self-Limiting Agricultural Pest Insect: Evaluating Its Potential for Future Crop Protection

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Alternative, biologically-based approaches for pest management are sorely needed and one approach is to use genetically engineered insects. Herein we describe a series of integrated field, laboratory and modeling studies with the diamondback moth, *Plutella xylostella,* a serious global pest of crucifers. A "self-limiting" strain of *Plutella xylostella* (OX4319L), genetically engineered to allow the production of male-only cohorts of moths for field releases, was developed as a novel approach to protect crucifer crops. Wild-type females that mate with these self-limiting males will not produce viable female progeny. Our previous greenhouse studies demonstrated that releases of OX4319L males lead to suppression of the target pest population and dilution of insecticide-resistance genes. We report results of the first open-field release of a non-irradiated, genetically engineered self-limiting strain of an agricultural pest insect. In a series of mark-release-recapture field studies with co-releases of adult OX4319L males and wild-type counterparts, the dispersal, persistence and field survival of each strain were measured in a 2.83 ha cabbage field. In most cases, no differences were detected in these parameters. Overall, 97.8% of the wild-type males and 95.4% of the OX4319L males recaptured dispersed <35 m from the release point. The predicted persistence did not differ between strains regardless of release rate. With 95% confidence, 75% of OX4319L males released at a rate of 1,500 could be expected to live between 3.5 and 5.4 days and 95% of these males could be expected to be detected within 25.8–34.9 m from the release point. Moth strain had no effect on field survival but release rate did. Collectively, these results suggest similar field behavior of OX4319L males compared to its wild-type counterpart. Laboratory studies revealed no differences in mating competitiveness or intrinsic growth rates between the strains and small differences in longevity. Using results from these studies, mathematical models were developed that indicate release of OX4319L males

should offer efficacious pest management of *P. xylostella.* Further field studies are recommended to demonstrate the potential for this self-limiting *P. xylostella* to provide pest suppression and resistance management benefits, as was previously demonstrated in greenhouse studies.

Keywords: biotechnology, engineered, insect, transgenic, *Plutella*

INTRODUCTION

Arthropod pests cause an estimated >$470 billion in lost agricultural crops worldwide (Culliney, 2014). The main tool for controlling such pests is the use of insecticides, the global annual market value of which is projected to reach $16.44 billion by 2019 (Statistica, 2019).

Insecticides will remain an important component of integrated pest management (IPM) programs but there are concerns about their off-target effects. Furthermore, resistance to insecticides is a growing problem, with 586 insect species known to be resistant to one or more insecticides (Sparks and Nauen, 2015). Other tactics will increasingly play a role in pest management in the future. Already the use of genetically engineered, insect-resistant crops (i.e., Bt crops expressing insecticidal proteins from the bacterium *Bacillus thuringiensis*) over the last two decades has played a major role in reducing the use of traditional insecticides in cotton, maize and other crops (James, 2017). As with traditional insecticides, however, the efficacy of Bt crops is threatened by the emergence of insects resistant to the Bt proteins expressed in them (Tabashnik and Carrière, 2017).

Genetic pest management goes beyond using genetically engineered pest-resistant crops and now includes genetic control of the pest itself. A predecessor of such methods is the sterile insect technique (SIT), in which sterile insects are released into wild populations of the same pest as a management intervention. This concept was independently conceived in the 1930s and 1940s by geneticist A. S. Serebrowskii in Moscow; tsetse field researcher F. L. Van der Planck in what is now Tanzania; and E. F. Knipling at the U.S. Department of Agriculture (USDA) (Klassen and Curtis, 2005). Van der Planck and Serebrowskii focused on sterility resulting from hybrid crosses between different species or different genetic strains. Knipling pursued the use of ionizing radiation to induce dominant lethal mutations causing the effect of sterility in treated insects (Knipling, 1955).

An early and on-going success has been the use of the radiation-based SIT against the New World screwworm, *Cochliomyia hominivorax*, a pest of livestock in the Americas (Gould, 2008). Decades-long international campaigns have suppressed and eradicated the New World screwworm from the USA and much of Central America and the Caribbean, with significant economic benefits (Vargas-Terán et al., 2005). A number of other pest insects have been successfully targeted by the SIT with associated reduction in the necessity for chemical control means; however, there are drawbacks to radiation-based SIT programs. The major limitation of many current SIT programs is that, in the absence of efficient sex-sorting methods, males and females are both released, which with many pests is likely to increase crop damage and/or reduce per-male efficiency (Rendón et al., 2004). Radiation can also have a negative impact on the performance of sterilized males in the field, reducing its economic feasibility (Bakri et al., 2005; Helinski et al., 2009). For many pest species, these factors prohibit use of the SIT.

Genetic approaches have been developed that overcome many of the limitations associated with SIT. One such strategy is the male-selecting, self-limiting genetic system that facilitates the mass-release of male-only cohorts of a given pest and avoids the use of potentially damaging radiation on the insects (Fu et al., 2007; Jin et al., 2013; Leftwich et al., 2014). In this system, colonies of a genetically engineered insect carry a transgene that confers female-specific mortality in the juvenile life stages, providing a means of mass-producing males which, after release into the field, find and mate with pest females. As carriers of the male-selecting, self-limiting gene, the female progeny of these released males cannot survive: with sustained releases of self-limiting males, females in the next generation are reduced, leading to population suppression. Provision of tetracycline (or suitable analogs) in the diet of larvae represses the engineered female mortality gene, allowing colonies of these insects to reproduce as normal to enable mass-production for large-scale application. Conversely, male carriers of this self-limiting gene survive as normal, even in the absence of tetracycline. Thus, after release of self-limiting males into the field, background, wild-type genetics from the mass-produced colony are introgressed into the target (wild) pest population via surviving male offspring. If the self-limiting colony comprises insects susceptible to Bt proteins (or to insecticides in general), studies indicate that sustained releases of self-limiting males can delay or even reverse the resistance developed in the target population to Bt proteins produced in genetically engineered crops (Alphey et al., 2007, 2009; Harvey-Samuel et al., 2015).

Diamondback moth, *Plutella xylostella,* is a global pest of crucifer crops estimated to cause losses of $4–5 billion annually (Zalucki et al., 2012). This species is a particularly damaging pest because of its high reproduction rate and its ability to develop resistance to most insecticides, including diamides and Bt proteins (Shelton et al., 1993; Talekar and Shelton, 1993; Zhao et al., 2006; Wang and Wu, 2012). In previous greenhouse studies with a self-limiting strain—called "OX4319L"—of *P. xylostella*, sustained introductions of self-limiting males into wild-type populations led to rapid population decline, then elimination (Harvey-Samuel et al., 2015). In the same greenhouse experiments using broccoli plants, relatively low-level releases of OX4319L males in combination with broccoli plants expressing Cry1Ac (Bt broccoli) suppressed pest population growth and

delayed resistance to Bt in the *P. xylostella* population (Harvey-Samuel et al., 2015). With the increasing threat of insect resistance to Bt crops, the application of self-limiting insects to delay or reverse the development of resistance, while providing pest control, demonstrates the compatibility of using these two types of genetic pest control (Alphey et al., 2007, 2009; Harvey-Samuel et al., 2015).

The promising results achieved with self-limiting *P. xylostella* suggest that further trials are justified. Herein we report results of open-field releases, with supplemental laboratory studies, assessing the performance of self-limiting *P. xylostella* and its potential as a biological control agent. Performance measures were selected as relevant to future operational deployment: field dispersal and persistence determining spatial and temporal release strategies and mating competitiveness and longevity. Good performance in these metrics will influence male mating effectiveness in the field and, therefore, efficacy of this vertically transmitted pest control strategy. Results from these field and laboratory studies were used to develop a mathematical model describing how releases of OX4319L males could reduce or prevent outbreaks of *P. xylostella* under field conditions.

The studies described here, conducted in New York State, represent the first open-field experiments with a self-limiting strain of an agricultural insect pest. Studies were conducted under a federal permit and state and university requirements. Data from the open-field releases provide empirical evidence of the persistence, survival, and distance traveled of OX4319L moths, compared to a wild-type strain, under conditions of the trials. These data will be useful from a management perspective, and for further testing or commercial use of this, or similar, strains of self-limiting insects.

Previous studies have been conducted in Arizona using a radiation-sterilized genetically engineered pink bollworm strain that, rather than carrying a self-limiting trait, carried a genetically-engineered fluorescent protein marker, as an addition to the SIT program against this agricultural pest (Simmons et al., 2011). Those studies were followed by multiple successful trials with a genetically engineered, self-limiting strain of the mosquito, *Aedes aegypti*—the primary vector of dengue, Zika, chikungunya, and yellow fever—in the Cayman Islands, Brazil, Panama and Malaysia (Harris et al., 2011, 2012; Lacroix et al., 2012; Carvalho et al., 2015; Gorman et al., 2016).

MATERIALS AND METHODS

Several sets of complementary studies, designed to compare biological parameters between the self-limiting strain of *P. xylostella*, OX4319L, and a wild-type strain, were conducted in open-field releases and in the laboratory. In each of the experiments, we compared aspects of the insect colonies described below. All experiments were performed at Cornell University's New York State Agricultural Experiment Station (NYSAES) in Geneva, NY during 2017–8, with field releases of OX4319L conducted in September 2017. Experiments were conducted under the USDA Animal and Plant Health Inspection Service, Biotechnology Regulatory Service permit 16-076-101r.

Insect Colonies

Two strains of *P. xylostella* were utilized for the tests and both were reared in separate walk-in environmental chambers set at 25°C on a 16:8 light to dark cycle. The OX4319L self-limiting strain of *P. xylostella* shows tetracycline-repressible, female-specific mortality: in the absence of tetracycline or suitable analogs in the larval diet, females die as larvae or pupae, whereas males survive as normal. On larval diet containing adequate concentrations of tetracycline, both sexes survive to adulthood. OX4319L insects also carry a DsRed2 fluorescent protein marker, which is visible under a fluorescence microscope in all life stages other than eggs, allowing personnel to visibly distinguish OX4319L insects from wild-type counterparts. The presence of the transgene can also be verified by PCR. The GA strain was captured from Omega Co., Georgia, USA in March 2014, and thereafter has been maintained in a laboratory on artificial diet for the larval stages. For the generation of male insects that were released, both strains were reared on diet that did not contain tetracycline (for male-only production of OX4319L) but did contain streptomycin.

Field Studies

Field Site

A field on the research farm managed by NYSAES was prepared according to standard practices. The chosen field was secluded from other crucifers on the farm and surrounded by woods on three sides. On 22–23 June 2017, cabbage (cv "Cabton") was transplanted into a field with the longest rows in the middle of the field and progressively shorter rows moving outward to create a circular field of 2.83 ha, with a diameter of 190 m. A 10 m buffer of bare ground was maintained around the perimeter of the circle. Plants were grown under standard practices until the release of the trial insects.

Field Release and Monitoring

Males of each strain, <24 h post-eclosion, were used for all releases. Prior to release, the sex of the moths was determined in the laboratory by examining adult genitalia while moths were anesthetized with CO_2. Moths were allowed to fully recover before being briefly anesthetized again to coat them with fluorescent powder (Day-Glo Corp., Cleveland, OH) and transferred to a 6-L plastic release container with lid (Berry Corp., Evansville, NC), in which they were held for 3–4 h before being released in the field.

Field Releases

Moths were released in the center of the 2.83 ha cabbage field by opening the container and allowing them to fly. Insects that did not immediately fly were placed on a 0.8 m high table and given more time to fly away. We conducted releases with different numbers of male moths to investigate whether the number of moths released from a given point may significantly affect their behavior. A total of six releases were made. One release of 1,000 moths of each strain was made in the evening of 8 September and two releases of 2,500 moths of each strain were made during the evenings of 12 September and 14 September. Both strains on each release date were coated with the same fluorescent-colored

powder to determine release date of recaptured moths; strain identification for each colored moth was verified by PCR. A single release of 1,500 and 1,000 moths, in which each strain was dusted with a different color, was made the evenings on 26 September and 27 September, respectively. On 28 September, 1,500 OX4319L and 1,400 GA males (the lower rate reflected available number of GA insects) coated with the same color were released. Strain identification for this release was also determined by PCR.

Recapturing Insects

Prior to releases, 48 traps were placed in the field in concentric circles at the following distances from the release site in the center of the field: 2 traps at 7 m, 4 traps at 14 m, 8 traps at 21 m, 10 traps at 28 m, 12 traps at 35 m, 4 traps at 55 m, 4 traps at 75 m, and 4 traps at 95 m. Traps in a given concentric circle were equidistant from each other. This design was developed based on a previous release of wild-type *P. xylostella* (the "Vero Beach" strain), in which ca 80% of moths that were recaptured in pheromone traps were captured 7–35 m from their release site (Bolton et al., 2019). Traps consisted of an inverted 355-ml Styrofoam cup, with a 3.3 cm-wide plastic rim at the base coated with Tanglefoot® (Olson Products Inc., Medina, OH), and were secured to fiberglass poles ∼0.5 m from the ground (just above the plant canopy) with a pheromone lure (Diamondback lure, Alpha Scents Inc., West Linn, OR) attached ∼2 cm above the trap. The trap design and layout were similar to previous studies conducted to monitor *P. xylostella* moths (Musser et al., 2005, Bolton et al., 2019). Each trap was collected and replaced daily after each release if any *P. xylostella* moths were present on it, until no marked *P. xylostella* were detected on any traps for 2 consecutive days. Due to rain that made the field inaccessible, no traps were collected on 29 September and 9 October, which corresponds to the days post-release for the following releases: days 3 and 13 for Release 4, days 2 and 12 for Release 5, and days 1 and 11 for Release 6, respectively. The fluorescent powder color (determined by visual inspection under UV light), trap location, and collection date were recorded for each recaptured moth. Individual moths were stored in 100% ethanol at 20°C for later PCR analysis.

Sample Identification

For the releases in which both strains were marked with the same fluorescent powder, PCR genotyping was used to identify the strain of each recaptured moth. For trap samples with more than 20 moths of a given color, 20 moths were randomly selected for PCR genotyping. Insect samples underwent PCR genotyping using the following conditions to verify that they were either OX4319L or wild-type.

To purify sample genomic DNA for PCR genotyping, we used two methods: either using a Purelink Genomic DNA kit (Invitrogen, Carlsbad, USA); or placing each sample in a Nunc Immuno Plate with 50 μL 300 mM sucrose solution (5.15 g sucrose, 0.875 g NaCl and 3 mL 1M Tris-HCl pH 8.0 in 50 mL ultrapure water), homogenizing the sample, incubating the sealed plate at 95–99°C for 9 min, spinning the samples at 4,000 rpm for 2 min, and placing samples on ice for 5 min before transferring the supernatant to a new 96-well plate. Each sample was genotyped by PCR [2 min at 95°C, 35 × (15 s at 95°C, 15 s at 62°C, 15 s at 72°C), and 5 min at 72°C] to detect two sequences, analogous to those described by Walters et al. (2012): one spanning the 5′ junction of the OX4319L genomic insertion (primers: "OX4319L-Pxy F2," sequence available on request; with "PB5-out," 5′-CTCTGGACGTCATCTTCACTTACGTG-3′); and the other spanning the wild-type locus of the same OX4319L genomic insertion site (primers: "OX4319L-Pxy F1"; with "OX4319L-Pxy R1," sequences available on request). Further PCR genotyping was undertaken on some samples, where the result of the first genotyping run was uncertain [2 min at 95°C, 40 × (15 s at 95°C, 15 s at 62°C, 15 s at 72°C), and 5 min at 72°C].

The identity of the remaining moths for that trap was estimated based on the proportion of each strain determined by PCR genotyping. For all cases where PCR genotyping failed, for instance due to inadequate genomic DNA template from a fragmented insect sample, the proportion of each strain was determined by the remaining samples associated with the same trap that were successfully genotyped. Trap collections from which none of the moths could be genotyped, either directly by PCR or indirectly by estimation (as described above), were not included in further analysis.

For each release (marked by a given powder color), the total of all the moths recaptured of each strain at each trap distance was determined for each day released (marked) moths were detected in the field. Each release had very high recapture rates at 7 m, indicating that released moths were over-sampled in traps at this distance; thus, all counts from 7 m were excluded from dispersal analysis but not from persistence analysis.

Persistence of Field-Released *P. xylostella*

To determine the persistence (i.e., how long released moths could be trapped in the field) of each strain in the field during each release, the relative cumulative proportion of moths recaptured (rc#R_p) each day for each strain within a release was calculated as follows: the cumulative number of moths of each strain recaptured daily (at all trap distances including 7 m) was divided by the total caught during that release to yield the cumulative proportion caught. This proportion was subtracted from the total number caught for that release to yield the relative cumulative proportion. To satisfy model assumptions, a $\log(\times + 0.001)$ transformation of rc#R_p was used for statistical analysis of persistence.

Survival of Field-Released *P. xylostella*

The daily sum of all moths recaptured at all trap distances (including 7 m) for each strain was calculated. This daily sum was divided by the number of moths initially released less those previously recaptured to yield the proportion surviving each day. The relative proportion surviving (rpS) is the daily proportion surviving divided by the proportion recaptured the first day post-release. Because some early daily observations were not undertaken due to rain (days 1 and 3 for one of the 1,500-moth releases and day 2 for one of the 1,000-moth releases) and the proportion recaptured after the first day post-release (the first day that moths were recaptured after release) was greater than the proportion recaptured on the first day post-release resulting

in the relative proportions surviving >1, only the data from the two 2,500-moth releases were used for analysis.

Mean Distance Traveled Calculation

The mean distance traveled (MDT) for each release was calculated according to the method of Morris et al. (1991) for each strain within each release rate using the number of moths recaptured at each trap distance > 7 m for each strain. The relative area for each annulus associated with each trap distance was calculated as the difference of the areas of a given (trap distance) anulus from the previous one divided by the area of the 14 m circle. The annulus distance used here was the distance of the trap from the release point at a given distance. For each release and day post-release, the cumulative number of each strain caught at each trap distance was determined by adding the number caught on the previous day to the current day for the entire monitoring period. The value from the last day monitored for a given release rate and strain was continued (unchanged) for the days up to the longest monitored period (14 days). These values were used to calculate the cumulative total estimated recapture (ER) and the product of the trap distance and cumulative total estimated recapture (ERXD) for each day post-release. To determine the daily MDT for each release and strain, the cumulative ERXD was divided by the cumulative ER for each day post-release. The overall daily MDT is the average of the daily MDT for each release. The MDT from the last day post-release is also the overall release rate for each release rate and strain. To satisfy model assumptions, $\sqrt{MDT}$ was used for statistical analysis.

Dispersal of Field-Released *P. xylostella*

To determine overall dispersal from the release point, the relative cumulative proportion of moths recaptured ($rc\#R_d$) at each trap distance beyond 7 m for each strain within release was calculated as follows: the cumulative number of moths of each strain recaptured at each trap distance was divided by the total caught during that release (excluding those caught at 7 m) to yield the cumulative proportion caught at each trap distance. This proportion was subtracted from the total number caught for that release to yield the relative cumulative proportion. To satisfy model assumptions, a $\log(\times + 0.001)$ transformation of $rc\#R_d$ was used for statistical analysis.

A separate analysis was conducted to determine the effect of strain and release rate on the proportion of moths recaptured relative to the number of moths released. For each release, the number recaptured at each trap distance for each strain was divided by the number of moths released for that strain (pR). To satisfy model assumptions, $\sqrt{(pR)}$ was used for statistical analysis.

Laboratory Studies

Mating Competition

The ability of OX4319L males to mate with GA females in competition with GA males was assessed using two methods: by determining the paternity of larvae of individual females throughout their lives, and by determining the paternity of larvae collected from a group of 20 GA females every 48 h up to 7 days. Only OX4319L males can pass on the fluorescent marker to their offspring, therefore the paternity of any larvae that showed DsRed2 fluorescence was assigned to OX4319L.

Competitive Mating With Individual Females

Two <24 h-old virgin GA females were placed with two <24 h-old GA males and two <24 h-old OX4319L males into each of six cages (60 × 60 × 47 cm) for 24 h. After this period, females were isolated individually to a 10 × 100 mm Petri dish containing an 18 × 18 mm coverslip treated with cabbage juice to induce oviposition. Thirty-one females were transferred every 48 h to a new dish with a freshly treated coverslip three more times (up to 7 days post-mating). To catch first-instars, each Petri dish was ringed on the inside with electrical tape (sticky side facing inward) to capture wandering larvae and covered. The number of eggs laid and the number of resulting larvae and fluorescent larvae were counted at 10× magnification using an Olympus SZX16 stereo microscope with a 100-W high pressure mercury burner (model #BH2-RFL-T3) and the combination of an Olympus SZX RFL3 filter with a barrier filter B580IF (to emit 520–550 nm light) within a week of exposure and recorded. The proportion of fluorescent larvae was recorded.

Competitive Mating With a Group of Females

Twenty 48 h-old virgin OX4319L and twenty 48 h-old virgin GA males were released into each of five 60 × 60 × 47 cm cages. After 4 h, twenty 48 h-old virgin GA females were released into the same cage. A 5 × 10 cm Parafilm sheet coated with cabbage juice was hung in the cage as an oviposition surface. This set up was replicated twice yielding 10 groups of females tested. Every 48 h the sheet with any *P. xylostella eggs* was collected and replaced, three more times. To catch first-instars, each sheet was placed in a 10 × 100 mm Petri dish ringed on the inside with electrical tape, as previously described, and covered. The dish was labeled with cage number, the date that the Parafilm sheet was exposed to the caged moths, and the days after the moths were released into the cages (day 1, 3, 5, and 7 post-mating). The number of eggs laid and hatched on the Parafilm sheet and total number of first-instars and DsRed2-positive larvae on the tape were counted at 10× magnification using the microscope described above. Counts to detect DsRed2 fluorescence were conducted within a week of exposure. The proportion of DsRed2-positive larvae was used for statistical analysis.

Intrinsic Growth Rate

To determine if mating with OX4319L males had any effect on the reproductive output of the females with whom they mated, the following experiments were performed. Twenty-eight <24 h-old virgin GA females were released into a cage with more than 500 GA males and 30 < 24 h-old virgin GA females were released into a cage with more than 500 OX4319L males, respectively, to mate for 2 h. Once mating was observed, females were placed individually in a 10 × 100 mm Petri dish with an 18 × 18 mm coverslip coated with cabbage juice. Females were transferred daily to a new similarly prepared Petri dish until death. Each dish was ringed with black electrical tape, as previously described, after the female was removed and covered. The numbers of

eggs laid and first-instars caught on the tape were recorded. The intrinsic growth rate (measured as larval output, not female progeny output) for each group was calculated as the sum of the products of the daily total number of larvae (m_i) and the proportion of surviving females (l_i) for each group according to the method of Wilson and Bossert (1971). The daily cumulative larval output ($\sum l_i * m_i$) was calculated by adding the previous daily larval output to the current day's larval output. To satisfy model assumptions, the square root of the cumulative daily larval output was used for statistical analysis.

GA and OX4319L Male Longevity

Two hundred < 24 h-old virgin males from each strain were collected from three different cohorts of pupae and divided equally between two treatments with one provided a 7.5% (w/v) sucrose solution with 67 mg/L methyl-4-hydroxybenzoate daily via a soaked cotton ball; the others were not. Each moth was individually contained in a 29.6 mL plastic cup with lid and checked daily until death. For each treatment, the proportion alive was calculated and used for statistical analysis.

The treatments for male and female longevity were arbitrarily assigned a number for analysis (Treatment 1, GA or OX4319L males not provided 7.5% sucrose solution; Treatment 2, GA female mated with GA or OX4319L male; Treatment 3, GA or OX4319L male provided 7.5% sucrose solution).

Statistical Analysis

JPM Pro 13.1.0 software (SAS Institute, Cary, NC; 2016) was used for all statistical analysis. Strain, release rate, release number, cage, replicate, female number and trap distance (for dispersal analysis relative to the number of moths released) were included in all analyses as categorical (nominal) variables. Days post-release and trap distance (for dispersal analysis relative to the number of moths recaptured used to estimate distances where 100 or 90% of moths were recaptured), days post-mating, day, and all response variables were analyzed as continuous variables. For all linear models and linear mixed effects models described below, a residual analysis plot (residual values vs. expected values) was visually examined to verify model assumptions of normality and homoscedasticity were met. Response variables were transformed as necessary to ensure that model assumptions were met. *Post-hoc* comparisons were made using Tukey's HSD method to control for multiple comparisons where $p < 0.05$ were considered statistically significant.

The overall percentage recaptured was analyzed using a linear mixed effects model with the proportion recaptured as the response variable and strain, release rate and their interaction as fixed effects and release number as a random effect.

Mean distance traveled was analyzed using a linear mixed effects model with $\sqrt{MDT}$ as the response variable and strain, release rate, days post-release and their interactions (full factorial) as fixed effects and release number as a random effect.

Dispersal data were analyzed using a linear mixed model with $\log(\text{rc\#R}_d + 0.001)$ and $\sqrt{(pR)}$ as response variables and strain, release rate, trap distance and their interactions (full factorial) as fixed effects and release number as a random effect.

Persistence data were analyzed using a linear mixed model with the $\log(\text{rc\#R}_p + 0.001)$ as the response variable and strain, release rate, days post-release and their interactions (full factorial) as fixed effects and release number as a random effect.

Field survival data were transformed using a Box Cox transformation [ln(rpS + 0.01)] to linearize the data and satisfy model assumptions. These transformed data were analyzed with a linear mixed effects model using ln(rpS + 0.01) as the response variable, strain and days post-release and their interaction as the fixed effects and release number as a random effect.

For both the individual and group mating competition experiments, the proportion of fluorescent larvae (p fl L) were analyzed using a linear mixed effects model with individual female number, and group number as random effects for individual and group experiments, respectively.

Data from the intrinsic growth experiment were analyzed using a linear model with the square root of ($l_i^* m_i$) as the response variable and mate, day and their interaction as fixed effects.

Longevity data were analyzed using a linear model with proportion alive as the response variable and strain, treatment, day and their interactions as fixed effects.

Population Modeling Studies

A deterministic model was developed to predict the effects of different release rates of OX4319L males on target populations in future suppression programs. The time horizon was 84 days (12 weeks) simulating about three generations during one cropping season given that egg-to-adult development time is 21 days in the model. Using findings from the mark-release-recapture studies described in this paper, we assumed equal daily survival rates (0.7/day) for adults of all genotypes. The number of adults, A, of sex s and genotype g on day t is

$$A(s,g,t) = I(s,g,t) + R(s,g,t) + 0.7A(s,g,t-1) + 0.225E(s,g,t-21)$$

where I is the immigration of adults, R is the number of released adults (only OX4319L males), and E is the number of adults maturing from eggs laid 21 days before. Note that A(s,g,0) = 0. In some simulations, a single immigration of wild-type *P. xylostella* occurs on the first day. In others, immigration of wild moths occurs once per week over the 84 days. Simulated releases of OX4319L males occurred either on only the first day, weekly, or every 2 weeks, with patterns sometimes matching the pattern of immigration.

Mating between males and females was assumed to be random, independent of moth genotype. Older females can mate more than once in the model. We assumed that female immigrants mate after arrival.

The dominant lethal allele kills all females except those homozygous for the wild-type. Because immigrants are homozygous for the wild-type allele, w, the only females that mate in the model are homozygous, and only released males, R, can be homozygous for the lethal allele. We account for this female mortality when calculating E(f,g,t), where f and m are designations for females and males. Fecundity was assumed to be 10 eggs/day and sex ratio of eggs was 0.5. Note that reproductive

TABLE 1 | Persistence (days) of two strains of *Plutella xylostella* released in a cabbage field.

Strain	Release rate	95% CI estimates* for days until			
		50% recaptured	75% recaptured	90% recaptured	100% recaptured
GA	1,000	(1.5–5.1)	(3.3–6.2)	(5.4–8.0)	(13.4–19.7)
OX4319L	1,000	(0.1–3.1)	(4.8–6.2)	(6.6–7.8)	(14.1–17.1)
GA	1,500	(0.6–1.6)	(1.6–4.1)	(3.5–5.5)	(11.0–15.1)
OX4319L	1,500	(3.5–5.1)	(3.5–5.4)	(5.4–7.1)	(12.7–17.9)
GA	2,500	(1.9–4.3)	(1.5–2.4)	(2.8–3.5)	(8.4–9.6)
OX4319L	2,500	(0.7–2.1)	(1.6–2.8)	(2.7–3.7)	(7.5–9.0)

Based on inverse intercept calculations using the regression equation for each strain and release rate combination with log(rc#R_p+0.001) as the response variable and days post-release as the only fixed factor (no random factors included). See **Table 2 for associated regression equations and predicted inverse intercepts.*

TABLE 2 | Regression equations and intercepts for persistence of *Plutella xylostella* strains released at three release rates.

Strain	Release rate	Regression equation	R^2 value	Predicted inverse intercept value (days)			
				50% recaptured	75% recaptured	90% recaptured	100% recaptured
GA	1,000	$\log(rc\#R_p + 0.001) = 0.562–0.0.225$*(days post-release)	0.64	3.7	5.0	6.8	15.7
OX4319L	1,000	$\log(rc\#R_p + 0.001) = 0.763–0.0.244$*(days post-release)	0.87	4.4	5.6	7.2	15.4
GA	1,500	$\log(rc\#R_p + 0.001) = 0.181–0.0.254$*(days post-release)	0.75	1.9	3.1	4.6	12.5
OX4319L	1,500	$\log(rc\#R_p + 0.001) = 0.478–0.0.237$*(days post-release)	0.76	3.3	4.6	6.2	14.7
GA	2,500	$\log(rc\#R_p + 0.001) = 0.100–0.0.348$*(days post-release)	0.95	1.1	2.0	3.1	8.9
OX4319L	2,500	$\log(rc\#R_p + 0.001) = 0.327–0.0.408$*(days post-release)	0.91	1.5	2.3	3.2	8.1

rate and mortality vary considerably in temperate North America (Harcourt, 1985; Dancau, 2018). Therefore, the values of E at time t for the viable sexes and genotypes are

$$E(f,w,t) = 10x0.5xA(f,w,t)x\,P(w)$$
$$E(m,w,t) = 10x0.5xA(f,w,t)x\,P(w)$$
$$E(m,y,t) = 10x0.5xA(f,w,t)x\,P(y)$$

Where the probability that mating produces a wild-type homozygote is

$$P(w) = [A(m,w,t) + 0.5A(m,y,t)]/[A(m,w,t) + A(m,y,t) + A(m,x,t)]$$

And the probability that mating produces a heterozygous male is

$$P(y) = [A(m,x,t) + 0.5A(m,y,t)]/[A(m,w,t) + A(m,y,t) + A(m,x,t)]$$

For genotypes that are homozygous wild-type w, heterozygous y, and homozygous x for the OX4319L allele. Again, the E(f,y,t) and E(f,x,t) all die, and no matings can create E(m,x,t) because of lack of A(f,x,t).

RESULTS

The results described below describe a series of studies evaluating the behaviors of the genetically modified self-limiting strain (OX4319L) compared to a wild-type counterpart (referred to as GA). Six open-field releases were performed at three different release rates, with two co-releases of both strains for each release rate. A series of laboratory studies compared the mating competence and longevity of the two strains. Results from the field and laboratory studies were utilized to generate a predictive deterministic model for calculating the ability of different release rates of OX4319L moths to suppress wild-type populations *of P. xylostella*.

Field Release Studies and Monitoring

Persistence of Field-Released *P. xylostella*

Persistence measured how long released moths were trapped in the field. Regression equations for $\log(rc\#R_p + 0.001)$ for each strain and release rate were used to calculate the expected mean time after release (days) and related 95% confidence intervals of when 50, 75, 90, and 100% of the male moths recaptured would occur (**Table 1**). These estimates varied with the release rate. For example, with 95% confidence, 90% of the 1,000 OX4319L males released that would be recaptured would be expected to be recovered after 6.6–7.8 days. With 95% confidence, 90% of the 1,500 OX4319L males released, that would be recaptured, would be expected to be recaptured after 5.4–7.1 days. With 95% confidence, 90% of the 2,500 OX4319L males released, that would be recaptured, would be expected to be recaptured between 2.7 and 3.7 days, respectively. In no cases did the persistence differ between the OX4319L and GA strains.

The difference in persistence between males from the 1,000- and those from 1,500-male release rates was not significantly different; however, the rate of persistence of both strains of males

TABLE 3 | Relative* percent survival of two strains of *Plutella xylostella* males released on six different dates.

			Days Post-release												
Strain	**Release rate**	**Release date**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
GA	2,500†	12-Sep-2017	100.0	40.4	38.2	21.1	1.8	1.1	1.1	0.7						
OX4319L	2,500†	12-Sep-2017	100.0	**111.4**‡	**145.5**	43.2	6.8	2.3	2.3	2.3						
GA	2,500†	14-Sep-2017	100.0	89.6	43.3	23.9	17.1	9.2	1.2	0.6	1.2					
OX4319L	2,500†	14-Sep-2017	100.0	50.7	39.0	6.0	18.1	9.1	0.0	0.0	0.0					
GA	1,000	8-Sep-2017	0.0	0.0	100.0	**140.7**	20.2	**101.3**	**101.8**	**225.2**	82.8	41.6	41.7	0.0	41.8	20.9
OX4319L	1,000	8-Sep-2017	0.0	0.0	100.0	**160.8**	81.1	**101.7**	40.9	*61.5*	20.6	*41.2*	0.0	*20.6*	0.0	0.0
GA	1,000	27-Sep-2017	100.0	—§	44.5	4.5	*36.0*¶	*27.2*	*18.3*	*27.5*	*9.2*	0.0	0.0	0.0	0.0	
OX4319L	1,000	27-Sep-2017	100.0	—	43.2	28.9	28.9	**275.3**	**310.2**	**437.9**	15.6	*62.4*	15.7	0.0	15.7	
GA	1,500	26-Sep-2017	100.0	15.2	—	0.0	0.4	1.5	*3.8*	*1.9*	*1.9*	0.0	0.4	0.0		
OX4319L	1,500	26-Sep-2017	100.0	58.4	—	8.2	1.2	*2.3*	*12.9*	*9.5*	*16.7*	*4.8*	1.2	1.2		
GA	1,400	28-Sep-2017	—	100.0	**150.9**	**418.6**	**584.8**	**551.2**	**930.0**	44.0	29.4	29.4				
OX4319L	1,500	28-Sep-2017	—	100.0	100.1	**1301.7**	**2929.1**	**4429.7**	**4033.8**	**327.3**	**1421.2**	**441.5**				

*Relative to the number collected on the 1st day that males of a strain were detected after a release date.

†Only data from 2,500 releases were used for survival analysis.

‡Value in **bold** because relative % survival was >100%.

§No data were recorded.

¶Value in italics because relative % survival was greater than previous day.

TABLE 4 | Field survival regression estimates of *Plutella xylostella* males at 2,500 release rate.

Term	Estimate	SE	*p*-value
Intercept	0.828665	0.229450	0.0010
Days post-release	−0.617303	0.042782	<0.0001

released at the 2,500-male release rate decreased significantly faster than those males released at a rate of 1,000 males ($P = 0.011$) and at a release rate of 1,500 males ($P = 0.007$) (**Table 2**). Persistence at the 2,500-male release rate was only 8.9 and 8.1 days for the GA and OX4319L strains, respectively, compared to >12 days for the other release rates.

Survival of Field-Released *P. xylostella*

Another measurement of the fate of the released insects is the relative proportion surviving (rpS) and is defined as the daily proportion surviving divided by the proportion recaptured the first day post-release. Moth strain had no effect on field survival ($P_{\text{strain}} = 0.4546$), but days post-release did ($P_{\text{days post-release}} < 0.0001$) (**Table 3**). These data fit the overall linear regression equation [ln(rpS + 0.01) = 0.828665-0.617303*days post-release] very well ($R^2 = 0.867$) (**Table 4**). This equation could be used to estimate field survival of future releases of OX4319L under similar conditions. This estimate would be conservative because the maximum time for moths released at the 2,500-male release rate persisted in the field was the shortest (8.1 days), relative to those released at the 1,000- and 1,500-male release rates (15.4 and 14.7 days, respectively) (**Table 2**).

Distribution of *P. xylostella* Recaptured in Field Releases

In the six mark-release-recapture studies, the percent recovered varied by distance from the release site for the GA and OX4319L strains (**Table 5**). For the closest trapping site (7 m), there was no significant difference in the overall mean percentage recovered ± SE for the GA strain and the OX4319L strain: 51.6% ± 8.1 and 47.8% ± 4.2, respectively. The combined percentage ± SE recovered between 14 m and 35 m was 46.2% ± 7.5 for GA and 47.6% ± 4.8 for OX4319L, respectively, with no significant difference. Thus, the total proportion recaptured in the first 35 m was 97.8 and 95.4% for GA and OX4319L, respectively. Less than 5% of released moths of either strain were recovered beyond 35 m, indicating the limited dispersal of both strains. Although no significant differences between populations were observed at any specific distance for any of the releases, the overall mean percentage recaptured from the six releases was significantly higher for the GA strain.

Mean Distance Traveled

The mean distance traveled by strain was highly variable for each release (**Table 6**). Although overall, OX4319L males (50.2 m ± 15.4, mean ± SE) traveled significantly farther than GA males (29.9 m ± 5.5) 14 days post-release (two-sample *t*-test, $P < 0.0001$), this appears to be due to the 1,500 release rate because no such statistical differences were observed with the other release

TABLE 5 | Percent recovered at different distances from the release point for two strains of *Plutella xylostella*.

Release Rate*	Release Date	GA recovered, 7 m	OX4319L recovered, 7 m	GA recovered, 14–35 m	OX4319L recovered, 14–35 m	GA recovered, >35 m	OX4319L recovered, >35 m	Overall recaptured GA	Overall recaptured OX4319L
1,000	8-Sep-2017	33.3	41.9	57.8	58.1	8.9	0.0	4.5	3.1
1,000	27-Sep-2017	30.0	43.3	70.0	44.4	0.0	12.2	6.0	9.0
1,500	26-Sep-2017	60.6	51.0	38.9	42.9	0.5	6.1	28.9	13.1
1,500†	28-Sep-2017	80.3	61.4	19.7	37.2	0.0	1.4	15.2	9.7
2,500	12-Sep-2017	64.1	56.0	34.1	37.3	1.7	6.7	23.2	3.0
2,500	14-Sep-2017	41.0	33.0	56.8	65.9	2.2	1.1	27.2	7.3
	Mean %‡± SE	51.6 ± 8.1	47.8 ± 4.2	46.2 ± 7.5	47.6 ± 4.8	2.2 ± 1.4	4.6 ± 1.9	17.5^{a} ± 4.3	7.5^{b} ± 1.6

**Number of male P. xylostella of OX4319L and GA released.*
† On 28-Sep-2017, 1,400 GA and 1,500 OX4319L were released.
‡ Mean % followed by different letters are significantly different as determined by a two-sample t-test, p = 0.0128.

TABLE 6 | Mean distance traveled [MDT] ±SE for *Plutella xylostella strains* released at three rates in field.

Release Rate		1,000		1,500		2,500		Overall*	
Days Post-		Strain		Strain		Strain		Strain	
Release	*N*	GA	OX4319L	GA	OX4319L	GA	OX4319L	GA	OX
1	12	9.8 ± 9.8	11.2 ± 11.2	30.0†	68.7†	32.9 ± 0.5	42.9 ± 21.5	23.1 ± 6.3	35.4 ± 13.3
2	12	9.8 ± 9.8	11.2 ± 11.2	14.0 ± 14.0	39.3 ± 25.3	34.3 ± 3.1	41.9 ± 1.3	19.4 ± 6.6	30.8 ± 11.0
3	12	20.0 ± 1.0	25.1 ± 2.9	14.0 ± 14.0	39.3 ± 25.3	43.4 ± 6.0	47.6 ± 13.4	25.8 ± 6.9	37.3 ± 8.5
4	12	19.7 ± 0.4	19.9 ± 2.3	21.0 ± 7.0	39.0 ± 25.0	42.9 ± 5.1	46.6 ± 13.2	27.9 ± 5.3	35.2 ± 8.9
5	12	19.6 ± 0.5	20.1 ± 2.1	21.0 ± 7.0	39.0 ± 25.0	44.1 ± 6.4	49.8 ± 7.8	28.3 ± 5.6	36.3 ± 8.7
6	12	20.2 ± 1.1	18.8 ± 0.1	21.6 ± 6.3	40.5 ± 23.4	44.1 ± 6.1	52.2 ± 10.5	28.6 ± 5.4	37.2 ± 9.1
7	12	19.4 ± 0.6	21.0 ± 2.6	22.8 ± 4.7	48.1 ± 13.7	44.0 ± 6.1	52.1 ± 10.6	28.7 ± 5.3	40.4 ± 7.7
8	12	26.6 ± 7.4	46.1 ± 26.6	23.0 ± 4.4	47.4 ± 13.9	43.9 ± 6.0	52.0 ± 10.7	31.2 ± 4.9	48.5 ± 8.3
9	12	26.3 ± 7.2	46.0 ± 26.4	22.8 ± 4.4	50.0 ± 9.5	43.8 ± 5.9	52.0 ± 10.7	31.0 ± 4.9	49.3 ± 7.8
10	12	26.2 ± 7.1	46.3 ± 25.9	22.9 ± 4.3	49.1 ± 9.5	43.8 ± 5.9	52.0 ± 10.7	31.0 ± 4.9	49.1 ± 7.7
11	10	25.7 ± 6.7	46.0 ± 25.7	26.2 ± 7.7	49.1 ± 9.5	49.8†	62.7†	30.7 ± 5.7	50.6 ± 9.2
12	10	25.7 ± 6.7	46.0 ± 25.7	26.2 ± 7.7	49.0 ± 9.4	49.8†	62.7†	30.7 ± 5.7	50.6 ± 9.2
13	6	25.1 ± 6.1	46.0 ± 25.7	33.9†	58.4†			28.1 ± 4.6	50.2 ± 15.4
14	6	27.9 ± 8.8	46.0 ± 25.7	33.9†	58.4†			29.9 ± 5.5	50.2 ± 15.4
Final MDT‡		27.9^{ab}± 8.8	46.0^{ab}±25.7	26.2^{a} ± 7.7	49.0^{b} ± 9.4	43.8^{ab}± 5.9	52.0^{ab} ± 10.7	29.9^{A} ± 5.5	50.2^{B} ±15.4

Traps at 7 m are not included and no observations were made after last value in column. Mixed model where $\sqrt{MDT}$ is the response variable; strain, release rate and days post-release are fixed variables and release number is a random variable. N = # releases × #strains × #release rates.
**Overall Strain means followed by different capital letters in last two columns are significantly different as determined by a two-sample t-test, p < 0.0001.*
† SE could not be calculated because data from only a single release was available.
‡ Final MDT values are from the last day post-release where observations from both replicates were made. Means followed by different small letters in bottom row ($HSD_{0.05,6}$ = 2.89) are significantly different.

rates. At the 1,500-male release rate, OX4319L males traveled significantly farther than GA males at the same release rate, 49.0 m ± 9.4 and 26.2 m ± 7.7, respectively) (Tukey $HSD_{0.05,6}$ = 2.89). At the release rates of 1,000 and 2,500, there were no statistical differences. These results are also shown graphically in **Figure 1A**, which illustrates the significant differences in distance traveled over time for all releases for both strains, while **Figure 1B** illustrates the differences by release rates and shows significant differences between the strains only at the 1,500 release rate.

Dispersal of Field-Released *P. xylostella*

For dispersal relative to the total number of moths recaptured, there was no significant effect of strain ($P_{\text{strain}} = 0.310$), release rate ($p_{\text{release rate}} = 0.685$) or the interaction between release rate and strain ($P_{\text{release rate*strain}} = 0.824$) on moth dispersal. Trap distance was the only factor that significantly affected moth dispersal ($P_{\text{trap distance}} < 0.001$). The overall regression equation $\log(\text{rc\#R}_d + 0.001) = 0.0099749–0.0327758*(\text{trap distance})$ accounted for 71% of the variation in the data ($R^2 = 0.71$) (**Table 7**). This equation was used to calculate the expected means, and related 95% confidence intervals, of the distances for each strain within a given release where 90 and 100% of the male moths recaptured would occur. With 95% confidence, 90 and 100% of what would be recaptured would be expected to be recovered between 25.8–34.9 m and 85.1–100.5 m from the release point, respectively. The high degree of confidence that

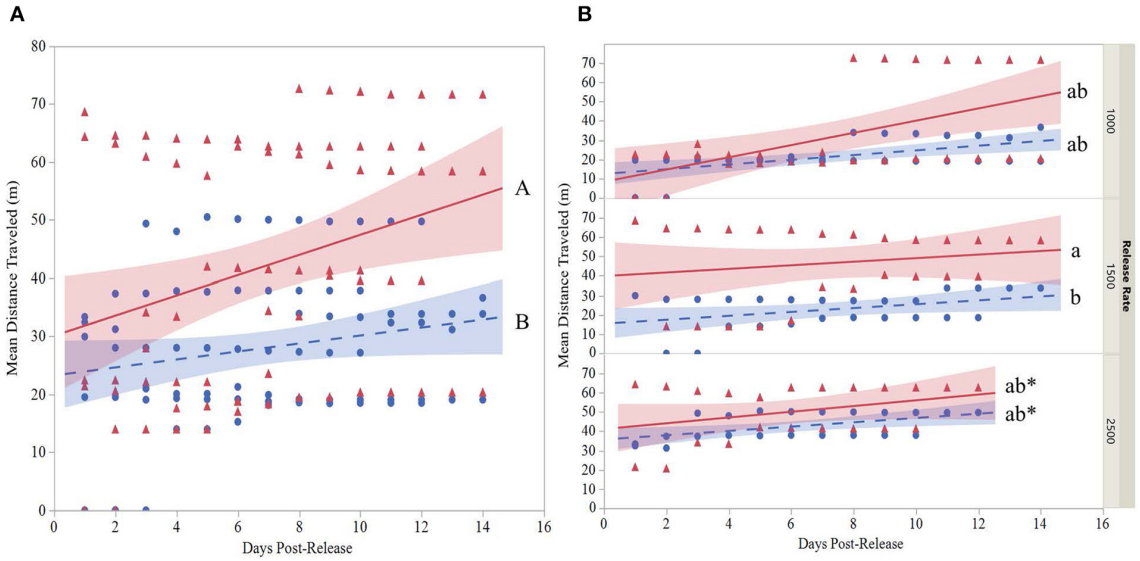

FIGURE 1 | (A) Overall regression with 95% confidence interval bands of the mean distance traveled (MDT) for two strains (- -●- -, GA; —▲—, OX4319L) of *P. xylostella* and **(B)** regression with 95% confidence interval bands at three release rates (1,000, 1,500, 2,500 males of each strain) in a 2.3 ha cabbage field (7 m traps excluded). MDT calculated according to Morris et al. (1991). Cumulative MDT analyzed with a mixed model where $\sqrt{MDT}$ is the response variable; strain, release rate and days post-release are fixed variables and release number is a random variable. *Last day observations were made at the 2,500 release rate. Overall strain lines with different capital letters (panel A) are significantly different as determined by a two-sample *t*-test, $P < 0.001$. Lines that do not share the same small letter(s) **(B)** are significantly different as determined by Tukey's HSD test, $HSD_{0.05,6} = 2.89$.

100% of the moths would be recaptured within 100.5 m of their release indicates that the 2.83 ha cabbage field was of appropriate size for the dispersal study.

For dispersal relative to the total number of moths released, significant differences between strains ($P_{strain} = 0.005$), the strain by release rate interaction ($P_{\text{strain*rel rate}} < 0.001$), trap distance ($P_{\text{trap dist}} < 0.001$) and the strain by trap distance interaction ($P_{\text{strain*trap dist}} = 0.0068$) were found (**Table 8**). At all distances except 14 m, there were no significant differences in the mean proportion recaptured. Few moths (≤0.1%) were recaptured at 55, 75 and 95 m. Overall distances, the recapture proportion was higher for GA (7.6%) than for OX4319L (3.7%).

Laboratory Studies

Mating Competition With Individual Females and With a Group of Females

In both scenarios, OX4319L showed similar mating performance to GA males. In both studies, all females produced a mixture of DsRed2-positive and DsRed2-negative larvae. Overall the mean (95% confidence interval) for the proportion of DsRed2-positive larvae (i.e., offspring of OX4319L males) was 52.0% (39.1–64.9%) and 57.3% (47.8–66.9%), when males of both strains were exposed to a group of two or 20 GA females, respectively. Because these confidence intervals include 50% (exactly equal mating competitiveness), mating competitiveness between OX4319L and GA are not significantly different. These results are similar to those seen in previous laboratory mating studies (Ant et al., 2012).

Intrinsic Population Growth

The intrinsic growth rate of a population, measured as the cumulative increase in the number of females from one generation to the next, was not affected by whether GA females mated with GA or OX4319L males ($P_{\text{mate}} = 0.2263$). The intrinsic growth rate (measured as the total number of larvae produced per generation) for GA females mated to OX4319L males was 920.9 and 1,031.5 for GA females mated to GA males. Although the cumulative number of larvae produced was not significantly different, the cumulative number of female progeny produced would be, because only the male larvae from GA females mated to OX4319L males would survive to adulthood.

Longevity

The longevity of each treatment group (males of either strain not provided with sugar water, males of either strain provided with sugar water and GA females mated to either strain and provided with cabbage juice to stimulate oviposition) was significantly different (Tukey $HSD_{0.05,3} = 2.35$) (**Figure 2**). However, for males not provided sugar water, and for females mated to males from either strain, the longevity of both strains was not significantly different. When provided with sugar water daily, OX4319L males lived significantly longer than did GA males (Tukey $HSD_{0.05,6} = 2.87$). However, for males not provided sugar water, and for females mated to males from either strain, the longevity of both strains was not significantly different. For GA females mated with OX4319L males or GA males, the median longevity was 8 and 10 days, respectively. The median longevity for GA males that were provided sugar water was 25 days. For

TABLE 7 | Regression equation and predicted intercepts values for dispersal of *Plutella xylostella* released in cabbage field.

Regression equation	R^2 value	Expected mean distance (m)		95% Confidence Interval (m)	
		90% recaptured	100% recaptured	90% recaptured	100% recaptured
log(rc#R_d + 0.001) = 0.0099775–0.0.032776*(trap distance)	0.71	30.7	91.8	(25.8–34.9)	(85.1–100.5)

TABLE 8 | Mean percentage ± SE of *Plutella xylostella* strains recaptured at distances from release point.

Strain*	Trap distance (m)							Overall Total†
	14	21	28	35	55	75	95	
GA	$3.3^{a} \pm 0.9$	$2.0^{ab} \pm 0.6$	$1.3^{bc} \pm 0.4$	$0.8^{bc} \pm 0.3$	$0.1^{de} \pm 0.1$	$0.0^{e} \pm 0.0$	$0.1^{de} \pm 0.0$	$7.6^{A} \pm 0.2$
OX4319L	$1.3^{bc} \pm 0.3$	$1.0^{bc} \pm 0.2$	$0.8^{bc} \pm 0.1$	$0.4^{cd} \pm 0.0$	$0.0^{e} \pm 0.0$	$0.1^{de} \pm 0.0$	$0.1^{de} \pm 0.0$	$3.7^{B} \pm 0.1$

*Strain means across trap distances for both strains followed by a different small letter are significantly different ($HSD_{0.5,\ 14} = 3.57$).
†Overall strain totals followed by different capital letters in last column are significantly different as determined by a two-sample t-test, $p = 0.0005$.

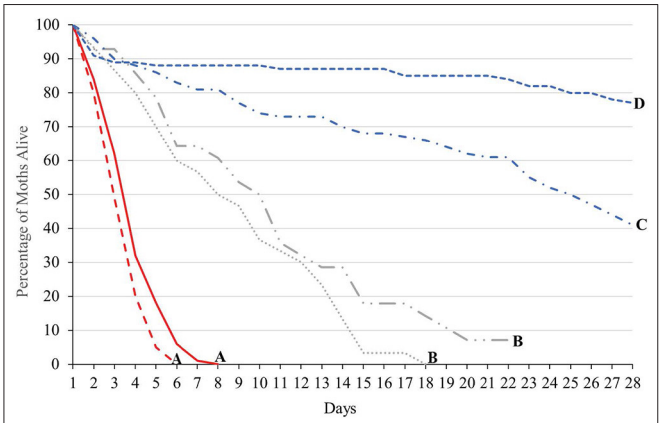

FIGURE 2 | Longevity of 2 strains of *P. xylostella* under 3 different conditions in the laboratory: 100 males (▬▬, GA; ——, OX4319L) not provided 7.5% sugar water daily; 100 males (▬·▬·▬, GA; ▬ ▬ ▬, OX4319L) provided 7.5% sugar water daily; 28 GA females (·····, mated to GA males) and 30 GA females (▬·▬·▬, mated to OX4319L males) from intrinsic growth rate study (see text). Lines with the same letter are not significantly different as determined by Tukey's HSD test, $HSD_{0.05,\ 6}$ =2.87.

the OX4319L males that were provided sugar water, 77% were still alive at 28 days. The median longevity of GA and OX4319L males without sugar water was 3 and 4 days, respectively.

Although the males supplied with sugar water lived longer than males without sugar water, this scenario is highly artificial and unlikely to be encountered under field conditions, where lifespan is anticipated to be significantly shorter. This increased longevity by OX4319L males is likely due to small differences in juvenile rearing conditions and/or adaptedness to laboratory rearing conditions: previous similar studies found the longevity of OX4319L males to be significantly lower than that of males from the same genetic background, reared under similar conditions (Jin et al., 2013).

Modeling Studies

Integrating the results from lab and field studies, predictive deterministic modeling indicates that bi-weekly releases of OX4319L males will effectively suppress populations of pest *P. xylostella* in the field (**Figure 3**). In temperate regions of the USA, where cold winters prevent year-round presence of *P. xylostella*, the pest immigrates from southern states early in the growing season. In one iteration of the model, in which a single influx of *P. xylostella* occurs—for example, on contaminated seedlings (Shelton et al., 1996)—an initial over-flooding rate as low as 2:1 (calculated as ratio of OX4319L released relative to number of immigrating wild males, with bi-weekly releases thereafter) prevented expansion of the *P. xylostella* population. In a second iteration, in which immigration of wild *P. xylostella* occurs more gradually over 3 weeks, the effective over-flooding rate was higher, with 25:1 achieving significant suppression (calculated relative to weekly number of immigrating wild males).

DISCUSSION

These studies describe the first open-field release of any self-limiting insect in North America, and the first open-field release of a self-limiting agricultural pest in the world. Overall, these results are significant because they provide empirical evidence of how far these transgenic moths traveled and persisted under the field conditions encountered during our trial. These results are similar to results from other field releases of wild-type *P. xylostella* moths. These results provide evidence of the expected persistence and spatial limitations of OX4319L moths in the field under similar conditions. From a management perspective, the results suggest that released OX4319L moths will largely remain in the area of the field into which they are released.

Overall, based on the number of moths trapped, the behavior of male OX4319L moths in the field was very similar to that of a strain (GA) collected from Georgia, in the southern USA, where *P. xylostella* is a perennial pest (Philips et al., 2014). Most importantly, at any given release rate the predicted persistence in the field did not differ between strains (**Table 1**). Both strains showed similar movement patterns in the field, with 94.2% (GA) and 95.4% (OX4319L) of the recaptured moths occurring within 35 m of the release point during the 2 week release period

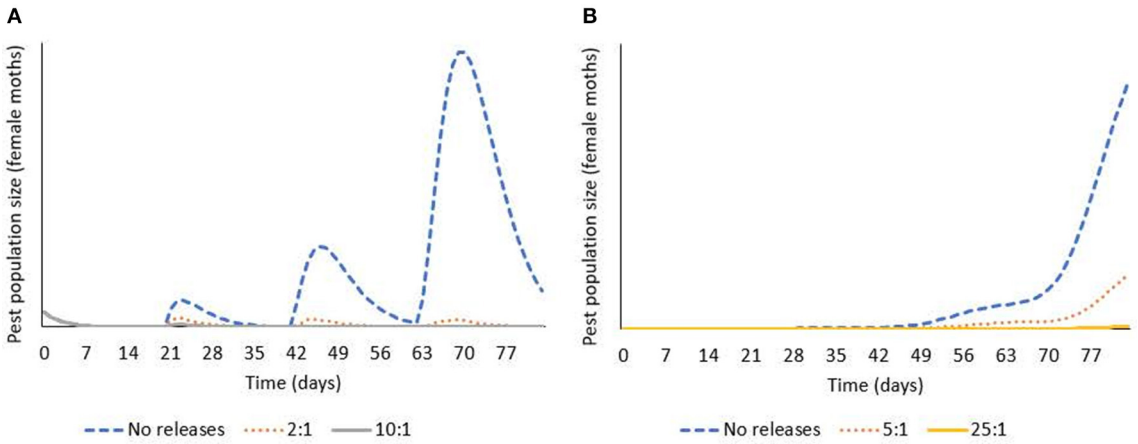

FIGURE 3 | Deterministic modeling shows releases of OX4319L males suppress pest populations of *P. xylostella*, in which bi-weekly releases of OX4319L male moths are conducted from day 0. **(A)** Response of pest population growth after a single immigration event (on day 0) to releases of OX4319L males at different initial over-flooding rates (2:1 and 10:1), and no releases of OX4319L males. **(B)** Response of pest population growth to gradual immigration (days 1–21) to releases of OX4319L males at different initial over-flooding rates (5:1 and 25:1), and no releases of OX4319L males.

(**Table 5**, **Figure 1**). Of the 10,000 OX4319L and 9,900 GA *P. xylostella* released during the entire study, < 1% of either strain was recaptured at 95 m (**Table 8**), suggesting that the field size was appropriate for these mark-release-recapture studies with *P. xylostella*. As a requirement stipulated by the USDA Animal and Plant Health Inspection Service for this study, pheromone traps were also placed outside the field with four traps placed at cardinal points at 0.25, 0.5, 0.75, and 1 km beyond the field border. No OX4319L moths were detected on any of these traps. A single GA moth was found 0.25 km beyond the field, suggesting that this strain's dispersal was also limited.

The proportion of OX4319L recaptured was very similar to that recaptured for a wild-type strain ("Vero Beach," the same genetic background as OX4319L) reported in a similar study by our program (Bolton et al., 2019), a study conducted prior to the field releases of OX4319L described here. Even though the present study deployed a higher number of pheromone-baited traps than in the previous study with the Vero Beach strain, it is worth noting that the transformed strain was recaptured in a distribution similar to its progenitor strain in these separate trials, again suggesting that adding the self-limiting trait did not affect its field behavior. The OX4319L males reared for this study exhibited similar eclosion and pupal rates and moths showed no obvious reduced activity in cages prior to release. Although a previous laboratory study found that the OX4319L transgene imposes a low fitness cost on the strain, our findings indicate that, as in a previous study (Somerville et al., 2019), in other experimental contexts this small fitness cost does not appear to significantly affect OX4319L male performance.

Additional confidence for our results with OX4319L is provided by a mark-release-recapture study of wild-type moths conducted in Australia (Mo et al., 2003). In that study the average distance traveled by *P. xylostella* before being recaptured on pheromone traps varied between 22 and 35 m over five releases. Although that study was conducted under different conditions—including trap design, crop and environmental conditions—the distance traveled by *P. xylostella* in that study is remarkably similar to the results from our study (**Table 8**).

Besides the distance traveled, it is also important to consider how long the moths persisted in the field. In the present study, both strains persisted in the field for a similar time when released at rates of 1,000 and 1,500 males per release and persisted significantly longer than those released at a rate of 2,500 males per release (**Table 1**). The reason for lower persistence at the higher rate remains unclear. The mean distances traveled by OX4319L and GA males were not significantly different at the 1,000 and 2,500 release rates but was different at the 1,500 release rate (**Table 6**). The reason for this difference also remains unclear. Most importantly, however, both strains were largely contained within the 2.83-ha field. In the context of evaluating the potential future field efficacy of OX4319L in a field release, our data suggest that OX4319L male dispersal was comparable with that of GA males.

Our field results indicate that, with 95% confidence, 75% of OX4319L males released at a rate of 1,500 could be expected to live between 3.5 and 5.4 days (**Table 1**) and 95% of these males could be expected to be detected within 25.8–34.9 m from the release point (**Table 7**). The mean distance traveled for OX4319L at this release rate was 39.3 m only 2 days after release (**Table 6**). For future suppression programs using OX4319L, these data suggest that releases, with either spatially continuous releases, or releases from discrete points 70 m apart, would provide appropriate coverage for every 0.25 ha of a given brassica field.

OX4319L and GA males had similar limited spatial dispersal and persistence under the conditions of this study. This is an important finding for implementation on farms. Control tactics, such as insecticide sprays, are usually deployed on a localized basis (e.g., a field). The site selected for the release was an isolated field surrounded by woods on three sides, including the side from which wind normally originates, and this may have helped

to limit moth movement beyond the field border. Additionally, no storms with increased winds occurred during the field tests that might have increased dispersal. Although there is evidence of long-distance movement of *P. xylostella,* primarily in high altitude winds (Talekar and Shelton, 1993), studies have indicated that movement within a suitable and stable habitat is limited (Mo et al., 2003; Musser et al., 2005; Bolton et al., 2019).

Our laboratory studies indicate that OX4319L males are equally competitive as GA males in mating with GA females. Such competitiveness is in contrast to SIT programs in which released males are usually less competitive and therefore have to be released at higher rates (Rendón et al., 2004; Bakri et al., 2005). Furthermore, in the present study both strains had similar lifespans under similar conditions, except OX4319L isolated males lived significantly longer than GA males when the former are given sugar water daily. The longevity of males without sugar water in the laboratory (6–8 days) is similar to the maximum survival of 8.8 days predicted by the regression equation for field survival. This 8.8 day survival in the field is very close to the 8.1 day field survival obtained in an Austrailan study (Mo et al., 2003). Another finding in the present study is that mating with OX4319L males does not appear to have any effect on the fecundity of wild-type females. Collectively these results indicate that, aside from female mortality in the absence of tetracycline in larval feed, the life history of the OX4319L strain is representative of unmodified *P. xylostella* moths, with no observed significant performance constraints.

Population modeling, incorporating data from these field and laboratory studies, indicates that sustained releases of the OX4319L strain (released twice per week, at initial over-flooding rates of 2–25:1 OX4319L males for every wild diamondback male moth in the target population) will lead to significant population decline over >3 generations (**Figure 3**). The model suggests that this pest management strategy can be flexible and adapted to a variety of invasion scenarios and different infestation levels while remaining efficacious. It should also be noted that these overflooding rates are lower than is typical for the SIT: for example, pink bollworm, *Pectinophora gossypiella* (60:1) (Walters et al., 1998); codling moth, *Cydia pomonella* (40:1) (Proverbs et al., 1982); and painted apple moth, *Orgyia anartoides* (100:1) (Suckling et al., 2002; Wee et al., 2005). Notwithstanding that efficacy-related field studies will provide more robust estimates of effective over-flooding rates with OX4319L, self-limiting insects are anticipated to offer improved performance compared to radiation-sterilized insects.

In addition to the control achieved by releasing the OX4319L strain alone, it should be noted that this biological control method can be combined with other biopesticides to achieve more sustainable management of *P. xylostella* populations. For example, our modeling did not account for the complementarity expected between, for example, releases of self-limiting male *P. xylostella* and application of the biopesticide, Bt, which targets the larvae of Lepidoptera while leaving adults unaffected. In the context of an integrated pest management (IPM) program, required release rates of OX4319L males are likely to‘be lower than modeled here due to the additional pest suppression effect provided by other modes of action, as indicated by previous glasshouse studies (Harvey-Samuel et al., 2015). In addition, releases of OX4319L would provide a resistance management benefit, where the efficacy of insecticides, such as Bt, is threatened (Alphey et al., 2007; Harvey-Samuel et al., 2015).

These integrated field, laboratory and modeling studies suggest promise for application of OX4319L for crop protection programs against *P. xylostella*. Further field studies are recommended to demonstrate the potential for this self-limiting *P. xylostella* to provide pest suppression and resistance management benefits, as previously demonstrated in greenhouse studies (Harvey-Samuel et al., 2015).

To be sustainable, agriculture needs to adopt a broader IPM approach to reduce reliance on insecticides. These results suggest this self-limiting strain may provide an effective management tool by itself on Brassica crops and improve the efficacy of chemical or plant-based insecticidal methods through resistance dilution.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article and accompanying files.

AUTHOR CONTRIBUTIONS

AS, SL, MB, and NM designed the study. AS, SL, AW, MB, HC, and LR performed the research. LJ, SL, and NM analyzed the data. NM constructed the model. AS, SL, LJ, and NM wrote the paper.

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REVIEW
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Regulation of Synthetic Biology: Developments Under the Convention on Biological Diversity and Its Protocols

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The primary international forum deliberating the regulation of "synthetic biology" is the Convention on Biological Diversity (CBD), along with its subsidiary agreements concerned with the biosafety of living modified organisms (LMOs; Cartagena Protocol on Biosafety to the CBD), and access and benefit sharing in relation to genetic resources (Nagoya Protocol to the CBD). This discussion has been underway for almost 10 years under the CBD agenda items of "synthetic biology" and "new and emerging issues relating to the conservation and sustainable use of biological diversity," and more recently within the scope of Cartagena Protocol topics including risk assessment and risk management, and "digital sequence information" jointly with the Nagoya Protocol. There is no internationally accepted definition of "synthetic biology," with it used as an umbrella term in this forum to capture "new" biotechnologies and "new" applications of established biotechnologies, whether actual or conceptual. The CBD debates are characterized by polarized views on the adequacy of existing regulatory mechanisms for "new" types of LMOs, including the scope of the current regulatory frameworks, and procedures and tools for risk assessment and risk mitigation and/or management. This paper provides an overview of international developments in biotechnology regulation, including the application of the Cartagena Protocol and relevant policy developments, and reviews the development of the synthetic biology debate under the CBD and its Protocols, including the major issues expected in the lead up to and during the 2020 Biodiversity Conference.

Keywords: synthetic biology, living modified organisms, Cartagena Protocol on Biosafety, Nagoya Protocol on Access and Benefit Sharing, risk assessment, gene drives, digital sequence information, biotechnology regulation

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INTRODUCTION

The world of "synthetic biology" is an optimistic and ambitious one, with its claims of transformative and paradigm-shifting developments, and promises of providing solutions for global challenges such as food security, energy security, clean water, human and animal health, environmental contamination, species conservation, and even climate change (Ro et al., 2006;

Khalil and Collins, 2010; Redford et al., 2013; Kelley et al., 2014; Organisation for Economic Cooperation and Development [OECD], 2014; Redford, 2014; Sliva et al., 2015; Crow, 2018; Gray et al., 2018). These promises appeal to research funders, fascinate the public and even inspire the next generation of scientists, however they also arouse fear in a society where biotechnology is often perceived as controversial. Whether or not "synthetic biology" could contribute toward these global challenges depends not only on the scientific realities matching the hype (Kwok, 2010; Cameron et al., 2014; Ostrov et al., 2019), but also the interconnected issues of the regulatory environment and societal acceptance.

Synthetic biology is part of the continuum of modern biotechnological development that commenced with the emergence of molecular cloning, recombinant DNA technologies and the polymerase chain reaction from the early 1970s and through the 1980s (Berg et al., 1974; Cameron et al., 2014). These technologies enabled the modification and intentional transfer of DNA from one organism into another and were perceived as truly paradigm-shifting. The developments in biotechnology in the 1970s were accompanied by both excitement and concerns about the potential risks. In response to the latter, in 1974 the scientific community recommended "voluntarily deferring" certain types of laboratory experiments until an international scientific discussion could be held to review scientific progress and examine the potential risks and how to manage them (Berg et al., 1974). In 1975 the Asilomar Conference on Recombinant DNA Molecules was attended by some 140 scientists, predominantly from public institutions from around the world, as well as lawyers, government officials and members of the media. At Asilomar it was agreed that the research should continue but with appropriate safeguards in place, thus heralding the beginning of precautionary biosafety regulation in this field (Berg et al., 1975; Berg and Singer, 1995; Berg, 2008).

The concerns about the risks of recombinant DNA and associated "new" technologies evident in the 1970s persist in present-day regulatory policy debates, and with the beginning of the current millennium there were calls for another "Asilomar" for "synthetic biology" (Brenner and Sismour, 2005). For those who witnessed developments in the 1970s, the current debates are a case of history repeating itself, with the same range of views expressed: from biotechnological developments being inherently risky and requiring stringent regulation based on the precautionary approach, through to these technologies not presenting unique or novel risks. The latter view is held predominantly by members of the scientific community who point out that much of "synthetic biology" is congruent with the technologies discussed in Asilomar in the 1970s, and that a substantial body of scientific evidence has accumulated over the past four decades with no documented hazard to public health attributed to products of these technologies (Berg and Singer, 1995; Brenner and Sismour, 2005; National Academies of Sciences Engineering and Medicine [NASEM], 2016a). However, in today's debate it is evident that concerns about the adequacy of regulation conflate broader political and societal issues beyond the safety of the technologies and their products.

At the international level, the United Nations Convention on Biological Diversity (CBD)[1] was one of several significant environment-related outcomes of the 1992 Earth Summit in Rio De Janeiro[2]. The CBD is ratified by 196 countries ("Parties"), which include all countries of the world except for the United States of America (USA) and the Holy See[3]. The objectives of the CBD (and its subsidiary treaties) are set out in **Figure 1**. During the drafting of the CBD, the potential for biotechnology to contribute to its objectives was recognized, provided that adequate safety measures were applied to its development and use (Secretariat of the Convention on Biological Diversity [SCBD], 2003). The resulting treaty obligates Parties to "regulate, manage or control the risks associated with the use and release of living modified organisms resulting from biotechnology which *are likely to have* adverse environmental impacts…" (emphasis added) [Article 8(g)]. It also provides the legal basis for a supplementary protocol [Article 19(3); see **Figure 2**] which CBD Parties started negotiating in 1995 (COP2; Decision I/9) and adopted in 2000 as the CBD's first subsidiary agreement, the Cartagena Protocol on Biosafety to the CBD ("Cartagena Protocol") (Secretariat of the Convention on Biological Diversity [SCBD], 2000). The Cartagena Protocol sets out a regulatory framework for the safe use, handling and transfer of living modified organisms (LMOs; analogous to genetically modified organisms/GMOs) (Secretariat of the Convention on Biological Diversity [SCBD], 2003). Some key provisions and definitions of the Cartagena Protocol that impact on the CBD synthetic biology debate are set out in **Figure 2**.

In addition to the Cartagena Protocol, the CBD has produced a second subsidiary agreement, the Nagoya Protocol on Access and Benefit Sharing to the CBD ("Nagoya Protocol") (Secretariat of the Convention on Biological Diversity [SCBD], 2011a), with the Cartagena Protocol also having a supplementary protocol on the topic of liability and redress (see **Figure 1**; Secretariat of the Convention on Biological Diversity [SCBD], 2011b). These two treaties are not the focus of this review, however they are relevant to the overall international biotech regulatory framework. The CBD and each of the Protocols have their own governing bodies, and since 2016 these have met in concurrent sessions during a 2-week "Biodiversity Conference."

At the time of writing (February 2020), several programs of work are in progress on various CBD and Protocol issues with relevance to synthetic biology, the outcomes of which will be considered by major meetings of CBD subsidiary bodies in May 2020 and the treaty governing bodies in October 2020 at the biannual Biodiversity Conference. Some of these issues are also under consideration as part of an extensive preparatory process underway for the development of the "Post-2020 Global Biodiversity Framework" that is expected to be adopted at

[1] *Convention on Biological Diversity*, Adopted 5 June 1992, 1760 UNTS 69 (entered into force 29 December 1993).

[2] See: https://www.cbd.int/history/

[3] See: https://www.cbd.int/information/parties.shtml

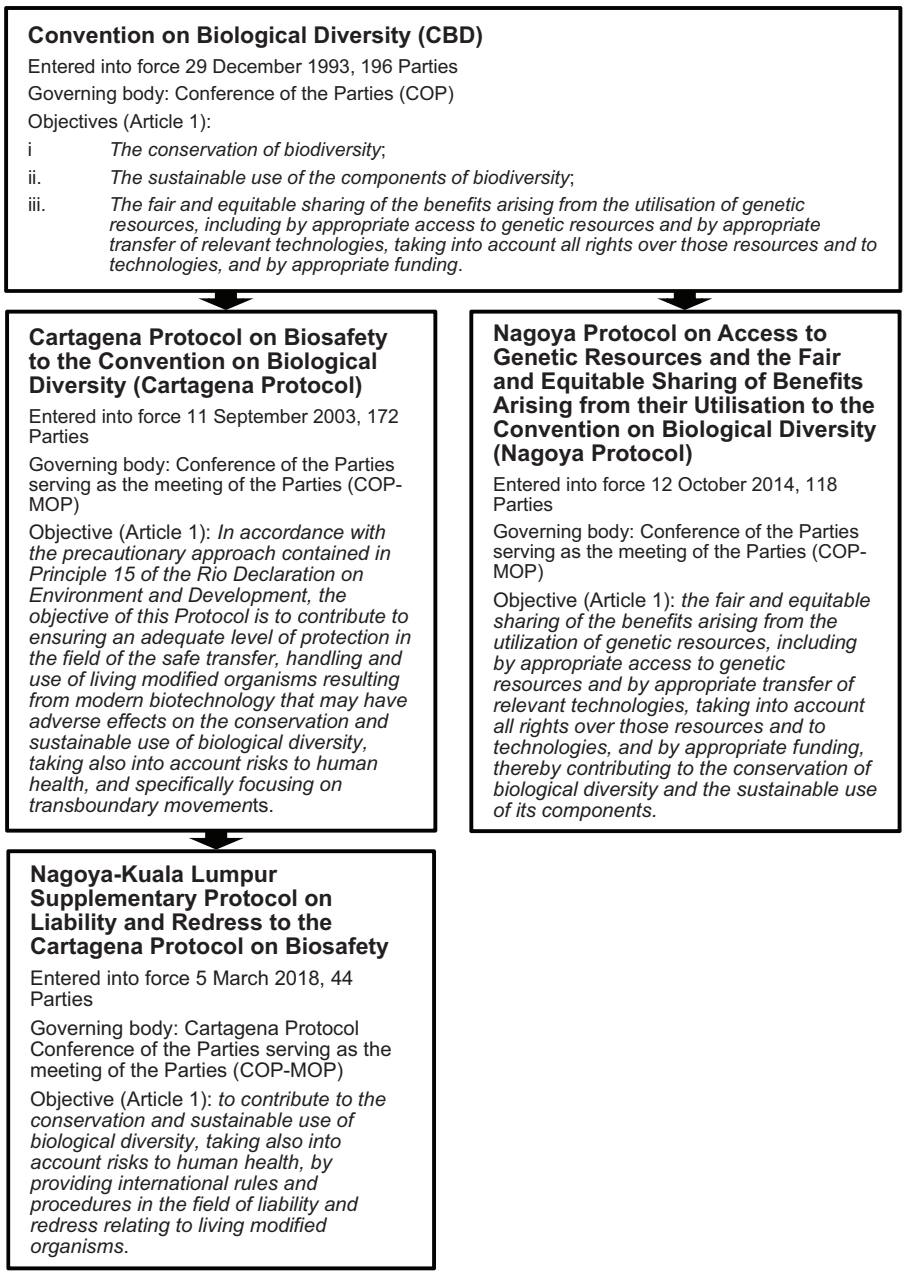

FIGURE 1 | The objectives of the CBD and its subsidiary treaties.

the Biodiversity Conference in October 2020. While synthetic biology is a CBD issue, it has overlap with other issues under the CBD's subsidiary protocols, as well as aspects of the Post-2020 Global Biodiversity Framework. This paper provides an overview of major developments in biotechnology regulation and relevant policy developments, examines what "synthetic biology" is, and reviews the development of the synthetic biology debate under the CBD and its Protocols, including the major issues expected in the lead up to and during the Biodiversity Conference in 2020. To begin, a brief overview is provided of the CBD treaty processes that form the basis of this discussion.

CBD PROCESSES – A PRIMER

To date, the CBD has generated 435 decisions at fourteen meetings of its governing body[4], the COP, and the fifteenth meeting of the COP (COP15) will be held in China in October 2020. The work of the COP is supported by two CBD subsidiary bodies: the Subsidiary Body on Scientific, Technical, and Technological Advice (SBSTTA)[5] and the Subsidiary Body

[4]See: https://www.cbd.int/decisions/

[5]See: https://www.cbd.int/sbstta/

Convention on Biological Diversity

Preamble – precautionary approach: *where there is a threat of significant reduction or loss of biological diversity, lack of full scientific certainty should not be used as a reason for postponing measures to avoid or minimise such a threat.*

Article 2 – Use of terms (definitions)

Biotechnology *means any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use.*

Genetic material *means any material of plant, animal, microbial or other origin, containing functional units of heredity.*

Genetic resource *means genetic material of actual or potential value.*

Article 19(3) – legal basis for the Cartagena Protocol

The Parties shall consider the need for and modalities of a protocol setting out appropriate procedures, including, in particular, advance informed agreement, in the field of the safe transfer, handling and use of any living modified organism resulting from biotechnology that may have adverse effects on the conservation and sustainable use of biological diversity.

Cartagena Protocol on Biosafety to the Convention on Biological Diversity

Article 3 – Use of terms (definitions)

*"**Living modified organism**" means any living organism that possesses a novel combination of genetic material obtained through the use of modern biotechnology.*

*"**Living organism**" means any biological entity capable of transferring or replicating genetic material, including sterile organisms, viruses and viroids.*

*"**Modern biotechnology**" means the application of:*

a. In vitro nucleic acid techniques, including recombinant deoxyribonucleic acid (DNA) and direct injection of nucleic acid into cells or organelles, or

b. Fusion of cells beyond the taxonomic family,

that overcome natural physiological reproductive or recombination barriers and that are not techniques used in traditional breeding and selection.

Article 10(6) – Decision procedure – precautionary approach

Lack of scientific certainty due to insufficient relevant scientific information and knowledge regarding the extent of the potential adverse effects of a living modified organism on the conservation and sustainable use of biological diversity in the Party of import, taking also into account risks to human health, shall not prevent that Party from taking a decision, as appropriate, with regard to the import of the living modified organism in question … in order to avoid or minimise such potential adverse effects.

FIGURE 2 | Key provisions of the CBD and the Cartagena Protocol.

on Implementation (SBI)[6]. The SBSTTA has met 23 times to date, with the twenty-fourth meeting (SBSTTA24) scheduled for May 2020, and synthetic biology is on the provisional agenda of that meeting[7].

Meetings of the SBSTTA may be described as "mini-COPs" because the outcomes from an increasing number of programs of work referred to it by the COP are deliberated and SBSTTA produces recommendations that become the basis (draft decisions) for the negotiations and subsequent decisions of the COP. For synthetic biology, SBSTTA24 is expected to address the COP14 request to *consider* the outcomes of a program of work that consists of submissions of information on a series of synthetic biology topics[8], a series of online discussions on those topics held in March 2019[9], and a report from the *Ad Hoc* Technical Expert Group (AHTEG) on Synthetic Biology that met in June 2019[10]. The SBSTTA24 is also requested to contribute to the completion of the analysis required to indicate whether or not synthetic biology qualifies as a "new and emerging issue" (NEI; Decision XIV/19). The latter refers to one of the functions of the SBSTTA to identify "new and emerging issues relating to the conservation and sustainable use of biodiversity," and this is discussed in further detail in section Synthetic Biology Under the CBD below.

[6]See: https://www.cbd.int/sbi/

[7]Item 4, document CBD/SBSTTA/24/1.

[8]See: https://bch.cbd.int/synbio/submissions/

[9]See: https://bch.cbd.int/synbio/open-ended/discussion/

[10]Document CBD/SYNBIO/AHTEG/2019/1/3.

The two Protocol COP-MOPs are also able to refer work to the CBD's SBSTTA, and for SBSTTA24 this includes the Cartagena Protocol topic of LMO risk assessment and risk management (Articles 15 and 16, Annex III), which has overlapping scope with synthetic biology[11]. An issue that arose from synthetic biology discussions that is now under consideration jointly by the CBD and the Nagoya Protocol is "digital sequence information" (DSI). This topic is under discussion within the Post-2020 Global Biodiversity Framework development process, which will also be on the agenda of SBSTTA24. All of these topics are discussed further in section Synthetic Biology Under the CBD below.

The COP and COP-MOP decisions, recommendations of the SBSTTA, reports of *Ad Hoc* Technical Expert Groups (AHTEGs), submissions of information, online discussions and NEI proposals that are referred to in this review are all available and accessible online via the CBD website[12]. Document, decision and recommendation numbers and weblinks are provided throughout.

THE CARTAGENA PROTOCOL

The Cartagena Protocol applies to any living organism that possesses a novel combination of genetic material *obtained using modern biotechnology* (emphasis added) (Article 3; see **Figure 2**). In other words, regulation of the organism is triggered by the use of modern biotechnology, which amounts to process-based regulation (Atanassova and Keiper, 2018). At the time the Cartagena Protocol and national and regional frameworks were drafted, process-based triggers may have provided a clear distinction between organisms within or outside of the scope of regulatory oversight. However, as biotechnology has evolved over time, distinctions have blurred and the continuing suitability of definitions developed in the 1980s and 1990s are questioned. For example, if the Cartagena Protocol's definition of "modern biotechnology" was strictly applied to take into account the need for overcoming "natural physiological or reproductive or recombination barriers and that are not techniques used in traditional breeding and selection," some recombinant DNA (e.g., cisgenesis) and "new" technologies (e.g., genome editing) may be excluded from its scope. Such definitions have given rise to debate in countries throughout the world on the regulatory status of "new techniques" such as genome editing, and this is one of the issues underlying the CBD discussions on synthetic biology (Atanassova and Keiper, 2018). In practice, Parties to the Cartagena Protocol differ in their interpretation and implementation of its definitions, with regulatory systems ranging from being largely process-based (e.g., European Union) to mostly product-based (e.g., Japan) (Nap et al., 2003).

Differences in implementation of the Cartagena Protocol also arise due to the primacy given to the precautionary approach which is introduced in the preamble of the CBD, and the Cartagena Protocol provides for its application in regard to making decisions about the import of LMOs [Article 10(6); see **Figure 2**]. It is a controversial feature of the Cartagena Protocol because it can lead to unpredictability in how biotech regulation is implemented; in highly risk-averse societies, the precautionary approach may be invoked to refuse the introduction of an LMO into the environment in the absence of identified risks if there is any doubt about its potential effects (Conner et al., 2003). A process-based trigger for LMO regulation is consistent with a precautionary approach, as its basis lies in the presumption that the technology is inherently risky, with all organisms resulting from biotech captured within regulatory scope regardless of their characteristics and the actual risks (if any) they present.

Another key feature of the Cartagena Protocol is the "advance informed agreement" procedure, which requires countries to be provided with the information necessary to enable them to undertake a risk assessment before deciding whether or not to permit the import of an LMO for intentional release into the environment (Articles 7, 10, 15, and Annex III). The principles of risk assessment set out in the Cartagena Protocol are found in biotech regulatory frameworks around the world, irrespective of whether or not the country is a Party to the Cartagena Protocol, as these principles were influenced by prior international guidance on the topic (e.g., Organisation for Economic Cooperation and Development [OECD], 1986, 1992, 1993) as well as the experience of countries already assessing LMOs for environmental release. Further, in practice any decision to release an LMO into the environment is informed by risk assessment, even when there is no transboundary movement associated with the release.

It should be mentioned that several countries who are major agricultural producers and exporters are not Parties to the Cartagena Protocol. Many countries developed biotech regulatory systems (e.g., Australia, Argentina, Brazil, China, the European Union, India, and South Africa) or adapted their existing legislative regimes (e.g., Canada and the USA) for the purpose of identifying and managing risks posed by GMOs to human health and the environment in parallel to the drafting of the CBD in preparation for the first releases of GM crops into the environment from the late 1980s and through the 1990s (Nap et al., 2003). Some of those countries became Parties to the Cartagena Protocol, but those that have not are still Parties to the CBD (except for the USA) and able to fully participate in discussions and decision-making and contribute their relevant expertise in the ongoing CBD synthetic biology discussions. These countries can participate in discussions under the Cartagena Protocol as "other governments" however they are not able to directly participate in decision-making under that treaty.

WHAT IS "SYNTHETIC BIOLOGY"

Synthetic biology is often reported to have emerged as a field of biotechnology in the early 2000s, with the convergence of engineering principles with biology (European Academies Science Advisory Council [EASAC], 2010). This time saw the first international meetings on "synthetic biology" and the beginning of the International Genetically Engineered Machine (iGEM)

[11] Item 5, document CBD/SBSTTA/24/1.

[12] See: https://www.cbd.int/

competition (Gray et al., 2018). It also saw the emergence of a new lexicon that reflected engineering concepts, e.g., standardized "parts" (such as "Biobricks"), chassis, circuits, plug-and-play; and claims of a new scientific culture of greater collaboration facilitated by the standardization of processes across labs, and openness and ethical awareness (Crow, 2018). The rapid adoption of the term "synthetic biology" may have been facilitated by its use by a self-defining new generation of researchers from a variety of disciplines (Oldham et al., 2012) seeking to differentiate themselves from past controversies, and also avoid issues with attracting funding and public acceptance. However, the high-profile publication of the first self-replicating bacterial cell with a fully synthetic "computer-designed" chromosome in 2010 (Gibson et al., 2010), which was accompanied by headlines proclaiming that man had created life[13], returned the spotlight on biotechnology regulation, and triggered new discussions on international regulatory oversight under the CBD.

Today many definitions and descriptions of synthetic biology can be found, but there are none that are universally agreed or applicable to everything that may be labeled as such in the CBD discussions. Fields or sub-areas of biotechnology that may be referred to as synthetic biology in the scientific literature and example applications are listed in **Figure 3**. At its simplest, synthetic biology may be described as combining DNA or genetic "parts" in novel configurations to modify existing properties or to create new ones (Oldham et al., 2012). More broadly, there is a general consensus that synthetic biology is a dynamic and growing area of biotechnology that utilizes accumulated and constantly advancing knowledge and understanding in biological engineering, and advancements in engineering tools (Raimbault et al., 2016). As for recombinant DNA technologies (Berg and Singer, 1995), synthetic biology is expected to have a profound impact on our knowledge of fundamental life processes. It is also expected to improve on and expand the range of potential biotechnological applications. Since the 1980s, recombinant DNA technologies have delivered products including drugs, industrial products and improved agricultural varieties (Berg and Singer, 1995). The focus of synthetic biology applications thus far include the production of pharmaceuticals and vaccines, and to provide alternatives to fossil-based fuels and relieve pressure on non-renewable resources (German Central Committee on Biological Safety [ZKBS], 2018; United Nations Environment Programme [UNEP], 2019).

While the use of the term synthetic biology is clearly broad and likely to evolve further with the advancement of technical and scientific knowledge, a challenge exists for regulators to distinguish what is truly new and not within the scope of existing applicable regulatory mechanisms. Identifying regulatory "gaps" is important as it allows regulations to be adapted to scientific progress. Distinctions that emerged early in the synthetic biology dialogue were based on expectations of unprecedented engineering complexity or scale, and speed (see the 2015 report of the AHTEG on Synthetic Biology[14]; European Academies Science Advisory Council [EASAC], 2010; Kuzma and Tanji, 2010). However, almost 10 years of work under the CBD (see section Synthetic Biology Under the CBD below) has failed to identify a LMO that would not be within the scope of the Cartagena Protocol, regardless of the complexity of the actual (or conceptual) genetic modification. In connection to the speed of development of the technology and the resulting organisms, countries have raised concerns about having the necessary resources to adequately assess and manage anticipated risks, however, regulators participating in the CBD discussions have not indicated that they have been inundated with such applications. More broadly, new types of LMOs may present resource and capacity challenges for less experienced regulators, and developing country Parties who have not yet, or are still in the process of, implementing the Cartagena Protocol at the national level.

Another often proposed distinction between recombinant DNA technology and synthetic biology is that the former involves the transfer of individual genes, whereas the latter involves the assembly of new DNA sequences (Science Communication Unit UWE, 2016). While this division is more technically specific, it remains an overly simplistic and inaccurate representation of recombinant DNA technologies as merely for "cut and paste," and of synthetic biology as a tool for generation of new DNA sequences. Both are based on common enabling technologies and involve the assembly of DNA sequences that are based on/are analogous to existing genetic material, and involve the transfer of genetic material into an existing living recipient cell/host. Thus, different views persist as to whether certain synthetic biology applications (particularly genetic circuits, metabolic engineering and genome synthesis; listed in **Figure 3**) are fundamentally new or are merely advances along the biotechnology continuum. There are examples of transgenic crops that are tagged by some with the synthetic biology label, particularly those that are examples of "metabolic engineering," e.g., Golden Rice that produces pro vitamin A, and crops that produce higher levels of omega-3 fatty acids. However, the promise of nutritionally enhanced crops, and work on developing Golden Rice, began with the dawn of plant genetic engineering in the early 1980s. The development of omega-3 crops followed in the 1990s (Enserink, 2008; Napier et al., 2019), with the first regulatory approvals in support of commercial cultivation of oilseed rape obtained in 2018[15]. Similarly, the design and construction of gene constructs using well characterized elements (referred to as "parts" by practitioners of synthetic biology), which are used in long-commercialized GM crops, are consistent with synthetic biology "approaches." Such innovative products may appear novel when they are ready for market, however their development may have taken 30 years (Napier et al., 2019).

Genome Editing

An area of technological development that is often linked with synthetic biology is the broad category of enabling tools for genome editing, in particular the technology known as "CRISPR" (clustered regularly interspaced short palindromic

[13] E.g., https://www.economist.com/leaders/2010/05/20/and-man-made-life

[14] Document CBD/SYNBIO/AHTEG/2015/1/3.

[15] See: https://www3.nuseed.com/us/nufarm-welcomes-worlds-first-regulatory-approval-for-plant-based-long-chain-omega-3/

i. The **design of genetic circuits** in living systems using components from different organisms. Example applications include biological sensors that respond to environmental stimuli such as certain pollutants, or metabolites in the human body (ZKBS, 2018).

ii. **Metabolic engineering**, which includes the design or redesign of a metabolic pathway by introducing several genes to an organism. Example applications include microbial (bio-factory) production of industrial chemicals, such as pharmaceuticals and biofuels, or microbial degradation of environmental pollutants (Pei et al., 2011).

iii. **Genome synthesis**, e.g. the first bacterial cell with an entirely synthesized genome was reported in 2010. This genome was designed by computer, chemically synthesized in the laboratory in yeast, and transplanted into *Mycoplasma mycoides* cells which exhibited expected phenotypic properties and were capable of self-replication (Gibson et al., 2010). Example applications include the design of chassis organisms for basic research and for biotechnological applications (ZKBS, 2018).

iv. **Minimal genomes (or cells)** whereby genetic material is removed in a top-down approach so that the genome contains only the genes that are essential for survival under certain defined conditions. In 2016, the design and synthesis of a minimal genome of the bacterium *Mycoplasma mycoides* was reported, with the 1079 kilobase genome reduced to 531 kilobases, consisting of 473 genes. Minimal cells may be used study the genetic requirements for life, for experimentation with genome synthesis, and to develop microbial platforms for performing new functions, e.g. the production of drugs or industrial chemicals (Hutchison et al., 2016).

v. **Protocells (or synthetic cells)** that are constructed in a bottom-up approach. These are cell-like vesicles assembled from non-living chemical components that may be designed to perform new functions (Pei et al., 2011). To date, protocells are not capable of replicating genetic material and are not considered to be living organisms (reports of the AHTEG on Synthetic Biology*)

vi. **Chemical synthetic biology (or xenobiology)** for the creation of orthogonal systems. This involves the use of altered or non-naturally occurring bases (xenonucleic acids) to expand the genetic code, or non-natural or non-canonical amino acids in polypeptides (Schmidt, 2010; Pei et al., 2011). An example application is the recent development of semi-synthetic interleukin-2 for the treatment of solid tumors, with evidence indicating the potential for reduced toxicity-associated side effects in patients (Synthorx, 2018). The incorporation of non-canonical amino acids is of increasing interest in the development of cell-free protein production systems (Zemella et al., 2015).

* Documents CBD/SYNBIO/AHTEG/2015/1/3, CBD/SYNBIO/AHTEG/2017/1/3, CBD/SYNBIO/AHTEG/2019/1/3.

FIGURE 3 | Sub-fields of biotechnology that may be referred to as synthetic biology in the scientific literature.

repeats) (Jiang and Doudna, 2017), and existing or conceptual applications of CRISPR such as organisms containing engineered gene drives (Legros et al., 2013), *de novo* domestication of species (Zsögön et al., 2018), and multiplex editing (Scientific Committees, 2014; Sánchez-León et al., 2017; Borrelli et al., 2018; Feng et al., 2018; Guo et al., 2018; Li A. et al., 2018; Li L. et al., 2018; Zsögön et al., 2018). Describing genome editing as synthetic biology is difficult to reconcile with the many descriptors of synthetic biology, particularly when considering the various potential outcomes of genome editing. For example, in crop breeding the outcomes of genome editing range from DNA sequence changes that are comparable to the outcomes of spontaneous or induced mutations, to targeted gene insertions which are comparable to transgenic crops (Custers et al., 2019). Therefore, in essence the outcomes of genome editing in crops are comparable to existing biotech and non-biotech approaches for generating genetic variation. Of note, in their assessment, three Scientific Committees advising the European Commission reported that multiplexed genome editing allows for genome-wide modification in a way that is more accurate and precise than changes made using conventional methods. They considered that it is the ease of using the technology and potential speed of development of new organisms that could present regulatory challenges in terms of adequate risk assessment (Scientific Committees, 2015b), rather than the technology or characteristics of the resulting organisms.

POLICY DISCUSSION ON SYNTHETIC BIOLOGY REGULATION

The initial developments in "synthetic biology" were mostly centered in the USA and in Europe, led by the United Kingdom, Germany, Switzerland and France. The USA remains the world leader in terms of research entities and investment in research and development (Pei et al., 2011). Expansion elsewhere in the world has been driven by the opportunities for investment in research and development, as well as for socio-economic development. This is evident for example in several countries in Asia that have invested in the establishment of national synthetic

biology initiatives that are contributing to advancements in the field, e.g., China, Japan, Korea, and Singapore (Chang, 2016; Ong, 2018).

The earliest synthetic biology policy discussions occurred in the USA, with similar timing to the beginning of synthetic biology discussions under the CBD, and these remain the most detailed investigations on technological developments under the umbrella of synthetic biology, their potential impacts and associated regulatory considerations. These are reviewed in brief below.

National Policy Developments

United States of America (USA)

With the 2010 publication of the first self-replicating bacterial cell carrying an artificially synthesized and assembled genome (synthetic genome), then United States President Obama requested that the Presidential Commission for the Study of Bioethical Issues examine the implications of this emerging science. The resulting report concluded that the science posed limited risks, and there was no justification for a moratorium, or the development of new federal regulations, i.e., the existing regulatory mechanisms applicable to "genetically engineered organisms" remained relevant. The report made the important observation that the work did not amount to "creating life," with this remaining a remote possibility for the foreseeable future; importantly, in this work the chemically generated genome was inserted into an already living naturally existing host cell (Presidential Commission for the Study of Bioethical Issues, 2010).

A series of reports followed from the National Academies of Sciences Engineering and Medicine (NASEM) addressing applications, products and enabling technologies that are included in the scope of "synthetic biology" CBD discussions. In their 2017 report on the "future products of biotechnology," the NASEM reached the conclusion that the ". . .scale, scope, complexity, and tempo of biotechnology products are likely to increase in the next 5–10 years. Many products will be similar to existing biotechnology products, but they may be created through new processes, and some products may be wholly unlike products that exist today." Such "similar" products include "next generation" GM crops, for which it was not anticipated that risk-assessment endpoints would be different from previously assessed GM crops. Less familiar products include gene drives designed to "suppress or enhance a species population at a rate that is faster than natural ecological processes or evolutionary rates"; such new products may require the definition of additional pathways to risk-assessment endpoints (National Academies of Sciences Engineering and Medicine [NASEM], 2017). These conclusions were broadly supported by NASEM reports published in the previous year that presented detailed reviews on the status of GM crops (National Academies of Sciences Engineering and Medicine [NASEM], 2016a) and gene drive research (National Academies of Sciences Engineering and Medicine [NASEM], 2016b). The NASEM emphasized the need for regulatory systems to have the agility to rapidly adapt to technological change and manage the assessment of a greater diversity of products (National Academies of Sciences Engineering and Medicine [NASEM], 2017).

The NASEM also examined the realistic capabilities of synthetic biology in the context of dual use. This is an issue long-connected to biotechnology that recognizes that while genetic engineering is predominantly pursued for beneficial purposes there is the possibility of it being applied for malicious use such as biological or chemical weapons. Their 2018 report on "biodefense in the age of synthetic biology" concluded that synthetic biology "expands the landscape of potential concerns," e.g., by modifying the properties of existing microorganisms, using microorganisms to produce chemicals, or employing novel or unexpected strategies to cause harm. The report recommended that the USA should closely follow advances in the field and develop expanded strategies to prevent and respond to emerging biologically-enabled threats (National Academies of Sciences Engineering and Medicine [NASEM], 2018). Of note, one such strategy under investigation is the "Insect Allies" project funded by a research agency of the US Department of Defense[16] which aims to use insects as a delivery tool for genetically modified viruses in order to address threats to food security by agricultural pests. This project has itself sparked dual use concerns amongst some researchers (Reeves et al., 2018), and concerns about new methods for "*in situ*" genetic modification in the CBD synthetic biology discussions.

United Kingdom (UK)

In the UK, a strategic roadmap (Roadmap) for synthetic biology was published in July 2012 (Technology Strategy Board, 2012) with the key purpose of developing "a roadmap that defines the likely timeframe and actions required to establish a world leading Synthetic Biology industry within the UK" (Clarke et al., 2012). The Roadmap was produced by an independent panel of experts for the government's Department for Business Innovation and Skills, and it sets out a vision for realizing the potential of synthetic biology with a focus on economic success, the use of cutting-edge science, and clear public benefit. While the Roadmap is primarily focused on recommendations for funding and policy activities to support research and innovation, it considers the applicable regulation and governance systems, and emphasizes the need for responsible research and innovation within an effective, appropriate and responsive risk-based regulatory framework. Notably, the Roadmap points out that synthetic biology "operates within the existing regulatory framework" for GMOs at the international (Cartagena Protocol), regional (applicable European Directives) and national (UK) levels, and the general consensus amongst regulators that these remain broadly adequate but a "watching brief" should be maintained as technology continues to develop.

In 2015, the UK Synthetic Biology Strategic Plan 2016 (Synthetic Biology Leadership Council, 2015) was released that built upon the 2012 Roadmap. It provided stronger focus on the responsible acceleration of commercial delivery of new products and services of public benefit and emphasized again the need for responsible research and innovation, and proportionate and adaptive regulation for the maximization of public benefit and minimization of risk. It also suggests

[16] See: https://www.darpa.mil/program/insect-allies

the development of technical standards at the national level to support the acceleration of commercialization (The British Standards Institution, 2015). These standards could also assist regulators, and support the UK in contributing to international discussions on appropriate regulatory and governance systems for synthetic biology.

Both the 2012 Roadmap and 2016 Strategic Plan briefly consider the issue of dual use, with the latter pointing out that guidelines and regulatory processes exist for accidental or deliberate misuse, that these are broader than synthetic biology, and that they need to be kept under review. The Strategic Plan also considers that synthetic biology tools have a key role in defending the UK against such incidents and regulatory systems need to enable rapid response.

Germany

Reports from other countries have been published more recently, with that by the German Central Committee on Biological Safety (ZKBS) in 2018 concluding that most synthetic biology approaches result in GMOs that can be assessed according to the existing German regulatory framework, the applicable European Directives (2001/18/EC and 2009/41/EC), and the Cartagena Protocol. Specifically, their assessment concluded that the insertion of synthetic genes, gene circuits, metabolic pathways, or entire genomes in an organism results in a GMO as defined by these regulatory frameworks. They also concluded that the reduction of a genome to create a minimal cell, and the use of xenonucleic acids to create bio-orthogonal systems are approaches that result in GMOs within the scope of existing regulatory frameworks. Further, they concluded that these developments did not present specific risks in addition to those already assessed for GMOs developed using recombinant DNA technologies (German Central Committee on Biological Safety [ZKBS], 2018).

Australia

Similarly, in October 2018 the final report produced following a review of the national regulatory framework by the Federal Government in Australia concluded that synthetic biology remains within its scope. In that review, synthetic biology was described as a "broad range of techniques, applications and products" that are "not qualitatively different" from that already regulated by the framework, but it was recommended that a "watching brief" be maintained to ensure this remained the case with future developments (Department of Health, 2018). This conclusion is consistent with the earlier advice of the Gene Technology Ethics and Community Consultative Committee – the committee that provides advice to the Office of the Gene Technology Regulator – in 2013 that synthetic biology did not raise new technical (or ethical) issues and was within the scope of the existing legislative scheme (Office of the Gene Technology Regulator, 2013). Also in 2018, the Australian scientific community reported the outcomes of a horizon scanning process, calling for the already progressive and effective regulatory framework to remain so, by timely responding to technological developments and ensuring regulation that is proportionate to risk (Gray et al., 2018).

Regional Developments

At the regional level, an early assessment by the European Academies Science Advisory Council (EASAC) in 2010 concluded that the regulatory frameworks that govern safe synthetic biology research and development are already in place or can readily be adapted to cope with the scientific advances foreseen (European Academies Science Advisory Council [EASAC], 2010). Mid-decade, a larger assessment was published by the European Commission in three "opinion" documents prepared by the Scientific Committees on Consumer Safety, on Emerging and Newly Identified Health Risks, and on Health and Environmental Risks. These opinions proposed an operational definition (Scientific Committees, 2014), examined the adequacy of European risk assessment practices for GMOs (according to the applicable regulatory framework) (Scientific Committees, 2015a), and research priorities (Scientific Committees, 2015b). The proposed operational definition coincided with work under the CBD on an operational definition (see section Synthetic Biology Under the CBD below), with that proposed by the Scientific Committees providing the basis for further elaboration by the CBD's AHTEG on Synthetic Biology in 2015[17]. Notably, on the topic of risk assessment, the Scientific Committees concluded that existing methodologies established for GMOs are adequate, and they made recommendations for research to improve knowledge for the purposes of risk assessment in regard to the particular developments they considered (genetic parts, minimal cells, protocells, xenobiology, DNA synthesis and genome editing, citizen science), and to ensure proportionate regulation with technological advancement (Scientific Committees, 2015a).

More recently in 2018, the European Commission has mandated the European Food Safety Authority (EFSA) to: (i) reflect whether and which newer sectors/advances should be considered among synthetic biology developments in addition to the six identified by the three Scientific Committees; (ii) to identify, where possible, potential risks in terms of impact on humans, animals and the environment for current or near future synthetic biology developments and to identify novel hazards as compared to established GMO techniques; (iii) to determine whether the existing European guidelines for risk assessment are adequate and sufficient for current and near future synthetic biology developments or whether there is a need for updated guidance; and (iv) in case guidance need to be updated, to identify the specific areas where such update is needed. While the publication of a final opinion after public consultation is expected by the end of 2020, the outcome of a literature search conducted by the German Julius Kühn-Institute as part of this work on synthetic biology developments in plants was briefly presented at an EFSA update meeting in June 2019[18], and it indicates that developments in the agri-food sector are "currently less advanced than in microorganisms" and that many scientists would not recognize plant metabolic engineering as a synthetic biology application.

[17] Document CBD/SYNBIO/AHTEG/2015/1/3.

[18] See: https://www.efsa.europa.eu/sites/default/files/event/4.5_Plant%20SynBio%20ERA_WG.pdf

International Developments

Organisation for Economic Development (OECD)

In recognition of the many potential applications of "synthetic biology" across a range of economic sectors, in 2014 the OECD published a report examining the associated policy issues (Organisation for Economic Cooperation and Development [OECD], 2014). This report highlights that the field benefits from the principles of risk assessment and "decades of regulation and governance" already developed for GMOs, with many experts considering that this is sufficient for synthetic biology as it is not significantly different from GM. It also points out that the potential benefits of synthetic biology may be hindered in some parts of the world due to over-regulation deterring investment in research and development (Organisation for Economic Cooperation and Development [OECD], 2014). An earlier OECD report examined the potential impact of developments in enabling technologies in biological sciences, including the then emerging field of synthetic biology, on industrial biotechnology. The combination of new enabling technologies with fermentation and biochemical engineering was considered to be a driver of economic development, however concerns regarding acceptance of GM were also recognized as a potential barrier to economic development (Organisation for Economic Cooperation and Development [OECD], 2011).

International Union for the Conservation of Nature (IUCN)

In the conservation biology field, some practitioners have expressed hope for a convergence between the traditional past-looking conservation mindset and the forward-looking optimism of synthetic biology, with speculation that it could contribute to saving endangered species and even reviving and restoring extinct species (Redford et al., 2013, 2014). Underlying this hope is recognition that new approaches and strategies are needed to address biodiversity loss that continues despite the application of conservation efforts. Applications of synthetic biology that are intended to have direct effects on biodiversity are therefore regarded by some as having great potential for addressing intractable conservation problems, such as the use of gene drives to control invasive species (Piaggio et al., 2017).

The optimism expressed by some is not shared by the all members of the conservation community, with some expressing deep concern (e.g., Civil Society Working Group on Gene Drives[19]). This led to a resolution at the 2016 IUCN World Congress to develop an IUCN policy on biodiversity conservation and synthetic biology[20], with a Task Force and Technical Subgroup on Synthetic Biology established to support this work. As a contribution toward the beginning of this process, the IUCN commissioned an assessment of the state of science and policy around synthetic biology techniques, including gene drives, as they relate to biodiversity, resulting in a recently published report (International Union for the Conservation of Nature and Natural Resources [IUCN], 2019). The assessment aimed to provide a clear understanding, based on the best available evidence, of synthetic biology issues that are relevant to and may have an impact on the conservation and sustainable use of biological diversity in order to inform future deliberations (International Union for the Conservation of Nature and Natural Resources [IUCN], 2019).

The IUCN report sheds light on tensions in the synthetic biology discussion in that forum that also exist under the CBD (see the following sections): polarized views on the safety versus danger of GMOs, and of their potential beneficial versus adverse effects on biological diversity. The report states that a major concern articulated by groups who are critical of conservation applications of synthetic biology is that they may serve as "Trojan horses" for other "more questionable" applications. In an attempt to address the topic without conflation of many different applications into one for adverse "summary judgment," the report takes a case-by-case approach and examines eight case studies with a conservation aim, or with a different aim but with impacts on conservation goals. The report also makes a plea for the policy debate to be grounded in evidence, emphasizing that conservation practice "needs to be rigorous and defensible, building on impartial standards that are free from ideology or political bias yet transparent in its advocacy for the natural world" (International Union for the Conservation of Nature and Natural Resources [IUCN], 2019).

The IUCN work on synthetic biology is running in parallel to the synthetic biology program of work under the CBD, and overlapping and cross-cutting programs of work under its Protocols. The IUCN holds a World Conservation Congress every 4 years, with the next one to be held in June 2020 where the draft IUCN synthetic biology policy will be brought to vote (International Union for the Conservation of Nature and Natural Resources [IUCN], 2019). The outcomes of the synthetic biology and gene drive discussions at the World Conservation Congress will likely have an influence on the CBD COP15 that will follow soon after in October 2020. This influence was evident in 2016, following the IUCN resolution calling for the synthetic biology assessment, as this resolution also called for gene drive research for conservation purposes to not be supported until this assessment was done. This was promoted as support for global moratorium on gene drive research in (so far unsuccessful) campaigns[21] for a COP decision supporting a moratorium on gene drive research (Callaway, 2016).

SYNTHETIC BIOLOGY UNDER THE CBD

The present status of "synthetic biology" in CBD discussions is that it falls within the CBD's broad definition of "biotechnology" (Article 2; see **Figure 2**), and "most organisms" developed or currently in development "through techniques of synthetic biology" are considered to be LMOs as defined by the Cartagena Protocol (Article 3; see **Figure 2**), and that for some organisms this may not be clear, such as "transiently modified organisms"

[19] See: http://www.synbiowatch.org/gene-drives-letter/

[20] Resolution WCC-2016-Res-086: Development of IUCN Policy on Biodiversity Conservation and Synthetic Biology. Available at: https://portals.iucn.org/library/node/46503.

[21] E.g., http://www.synbiowatch.org/gene-drives-letter/; http://www.etcgroup.org/sites/www.etcgroup.org/files/files/cbd_cop_13_gene_drive_moratorium_briefing.pdf

and those developed using certain applications of genome editing (see the 2019 report of the AHTEG on Synthetic Biology[22]).

These are the conclusions drawn from an extensive body of work that began in 2010 (see **Figure 4**) and is ongoing. In 2011 a group of civil society organizations called for urgent consideration of synthetic biology via the CBD's mechanism for proposing NEIs. These proposals claimed absent or insufficiently comprehensive regulatory oversight, or inadequate regulatory mechanisms for assessing risk, and called for bans on environmental releases and commercial approvals of LMOs developed via synthetic biology until risk and adequacy of regulatory oversight were examined (e.g., EcoNexus[23]; ETC Group[24]; International Civil Society Working Group on Synthetic Biology[25]).

At COP11 that followed these first NEI proposals, and all subsequent COPs, Parties and other governments have been urged to apply a "precautionary approach" with synthetic biology (COP11, Decision XI/11; COP12, Decision XII/24; COP13, Decision XIII/17) or gene drives (COP14, Decision XIV/19), reflecting the preambular language of the CBD (see **Figure 2**). The CBD synthetic biology program of work began with the COP11 decision in 2012 inviting submissions of information addressing the criteria for identifying a NEI (Decision XI/11). These criteria were established by a prior COP and are set out in **Figure 5** (COP9 in 2008; Decision IX/29). The information collected from these submissions was considered at a subsequent meeting of SBSTTA (SBSTTA18) which concluded there was insufficient information to finalize an NEI analysis for a decision on whether or not synthetic biology is a NEI (Recommendation XVIII/7; Decision XII/24). There has not been consideration of synthetic biology against the NEI criteria by a meeting of the SBSTTA since then, however this is expected to be reconsidered at SBSTTA24 in May 2020. If SBSTTA24 makes a recommendation on the topic, it will be followed by deliberation and a decision by the CBD Parties at COP15 in October.

At COP12 in 2014, the CBD Parties decided, in addition to further submissions of information on synthetic biology, to establish an "open-ended online forum" for a series of discussions on synthetic biology topics, and to establish an AHTEG on Synthetic Biology to deliberate all of the information received and make recommendations for consideration by the next meeting of the SBSTTA (Decision XII/24). This marked a significant expansion of the CBD's synthetic biology program of work despite there being no recommendation from a SBSTTA meeting or a COP decision that it is in fact a NEI requiring such attention. The program of work is notable for at least two reasons; firstly while the decision highlighted that the Parties "await" a "robust assessment" of synthetic biology against the NEI criteria, it did not attempt to directly address these. Secondly, it mandated the AHTEG to develop an "operational definition" (see **Figure 6**), the outcome of which was controversial and never formally adopted or endorsed by the CBD Parties at SBSTTA20 (IISD, 2016a) or COP13 (IISD, 2016b). The COP13 decision in 2016 "acknowledges" it as an outcome of the work of the AHTEG that is considered to be a "useful as a starting point for the purpose of facilitating scientific and technical deliberations" under the CBD and its Protocols (Decision XII/17). Despite the dissatisfaction with the definition, it has not been addressed again in subsequent synthetic biology work programs.

The incomplete NEI analysis is another point of contention for some Parties, who have increasingly expressed their dissatisfaction in recent meetings of the SBSTTA where synthetic biology was on the agenda (e.g., SBSTTA20, IISD, 2016a; and SBSTTA22, IISD, 2018). The COP decisions that followed called for further online discussions and submissions of information, and extensions of the AHTEG on Synthetic Biology in programs of work that largely expanded on or duplicated the topics that were deliberated in the previous intersessional periods. However, the terms of reference for the AHTEG on Synthetic Biology in those decisions included the NEI analysis: the COP13 decision in 2016 included an "analysis" by the AHTEG against the NEI criteria (Decision XIII/17), and the COP14 decision in 2018 required the AHTEG to "provide advice" on the relationship between synthetic biology and the NEI criteria (Decision XIV/19).

At the AHTEG on Synthetic Biology meeting in December 2017 (following COP13) the mandated NEI "analysis" was deferred pending clarification from the SBSTTA on how the NEI criteria should be applied. This topic was on the agenda of the subsequent SBSTTA21, but no recommendations were made (Recommendation XXI/7). The CBD Secretariat then prepared a document titled "Analysis against the criteria set out in paragraph 12 of decision IX/29," whereby text was taken from the AHTEG meeting reports from 2015 and 2017 and allocated to the NEI criteria where it was considered to have relevance. This document was controversial given that the AHTEG's deliberations and reports did not specifically address these criteria, hence the text used by the Secretariat could have been taken out of context, and not reflect the views of the entire AHTEG on Synthetic Biology[26]. At the AHTEG on Synthetic Biology meeting of June 2019 (following COP14), each of the paragraph 12 criteria were finally deliberated. The criteria were also included in the topics for submissions of information and the online discussions in the broader program of work. The expectation that the SBSTTA24 in May 2020 will revisit this outstanding analysis is based on this collection of information directly addressing the criteria.

Despite the formal NEI process being bypassed to date, synthetic biology has become a fixture on the CBD agenda, particularly since COP12, where there has been extensive debate about the adequacy of existing regulatory oversight for biotechnology and its potential positive and potential negative impacts. A criticism of this debate include its focus on hypothetical applications of "new" technologies

[22] Document CBD/SYNBIO/AHTEG/2019/1/3.

[23] Available at: https://www.cbd.int/doc/emerging-issues/econexus-synthetic-biology-2011-013-en.pdf

[24] Available at: https://www.cbd.int/doc/emerging-issues/etcgroup-introduction-synthetic-biology-2011-013-en.pdf

[25] Available at: https://www.cbd.int/doc/emerging-issues/Int-Civil-Soc-WG-Synthetic-Biology-2011-013-en.pdf

[26] E.g., see the online discussion "Topic 7: Relationship between synthetic biology and the criteria set out in decision IX/29," held 25-31 March 2019. Available at: https://bch.cbd.int/synbio/open-ended/discussion/.

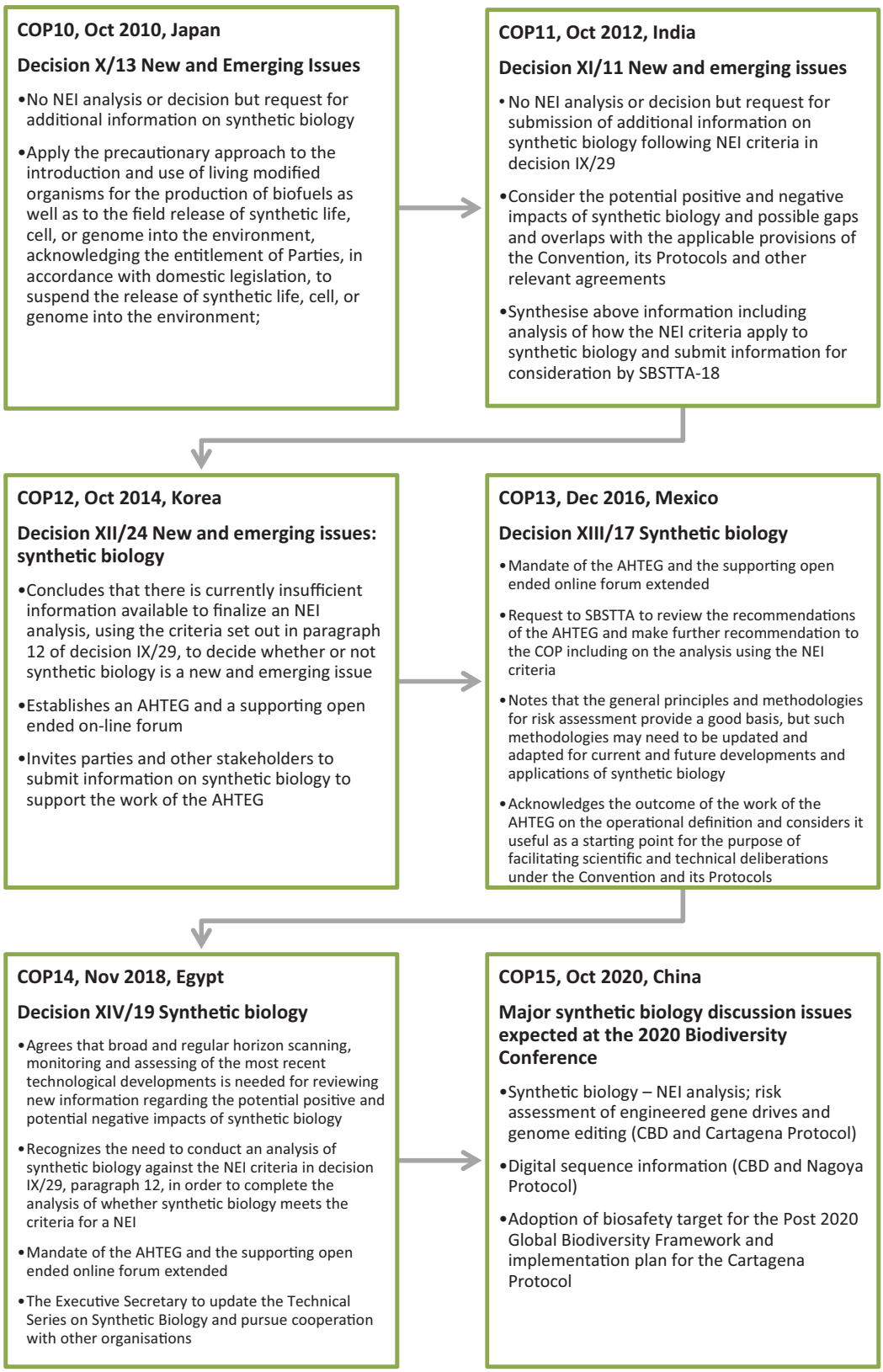

FIGURE 4 | Timeline and highlights of synthetic biology and related NEI COP decisions.

New and emerging issues relating to the conservation and sustainable use of biological diversity

The Conference of the Parties:

Article 11 *Decides that proposals for emerging issues should, where possible, be accompanied with information on:*

a) Why the issue needs urgent attention by the Subsidiary Body on Scientific, Technical and Technological Advice (including how it impacts biodiversity);

b) How it affects the attainment of the objectives of the Convention (citing relevant articles);

c) Thematic programmes of work and/or cross-cutting issues that could contribute to the resolution of the issue;

d) Work already under way by relevant organizations addressing the issue; and

e) Credible sources of information, preferably from peer-reviewed articles;

Article 12 *Further decides that the following criteria should be used for identifying new and emerging issues related to the conservation and sustainable use of biodiversity:*

a) Relevance of the issue to the implementation of the objectives of the Convention and its existing programmes of work;

b) New evidence of unexpected and significant impacts on biodiversity;

c) Urgency of addressing the issue/imminence of the risk caused by the issue to the effective implementation of the Convention as well as the magnitude of actual and potential impact on biodiversity;

d) Actual geographic coverage and potential spread, including rate of spread, of the identified issue relating to the conservation and sustainable use of biodiversity;

e) Evidence of the absence or limited availability of tools to limit or mitigate the negative impacts of the identified issue on the conservation and sustainable use of biodiversity;

f) Magnitude of actual and potential impact of the identified issue on human well-being;

g) Magnitude of actual and potential impact of the identified issue on productive sectors and economic well-being as related to the conservation and sustainable use of biodiversity;

FIGURE 5 | The NEI criteria from COP9 Decision IX/29.

rather than actual or realistically foreseeable and technically plausible applications (e.g., see CBD submissions by the Global Industry Coalition[27]). For example, the 2019 meeting of the AHTEG on Synthetic Biology considered "synthetic biology applications that are in the early stages of research and development," and the meeting report[28] includes a list compiled from various sources such as research proposals (e.g., environmental applications of engineered bacteria, Gumulya et al., 2018), early stage research reports (e.g., engineering coral, Cleves et al., 2018), and first demonstrations of technology (e.g., gene drive mechanisms in a mammal, Grunwald et al., 2019; and an agricultural pest, Buchman et al., 2018). Another criticism is that demands for expansion of risk assessment requirements disregard the existing experience and accumulated knowledge regarding LMO risk assessment, and the existence of biotech regulatory frameworks and other applicable regulatory mechanisms at international, regional and national levels (CBD submissions by the Global Industry Coalition). The current major issues expected to be debated at the upcoming CBD meetings in 2020 are detailed in the following section.

MAJOR SYNTHETIC BIOLOGY ISSUES AT THE 2020 BIODIVERSITY CONFERENCE

As referred to above, in October 2020 the governing bodies of the CBD (COP15) and its Protocols (COP-MOP10 for the Cartagena Protocol; COP-MOP4 for the Nagoya Protocol) will meet in concurrent sessions held over a 2-week period in Kunming, China. Synthetic biology is a stand-alone agenda item under the CBD, and it will also be considered within the NEI agenda item. Deliberations on these two agenda items will be based on draft recommendations produced at SBSTTA24 in May 2020.

New and Emerging Issue Analysis

If the outstanding NEI analysis is addressed at SBSTTA24 as anticipated, and is then the subject of a decision at COP15, this is expected to be one of the most contentious CBD synthetic biology topics at the 2020 Biodiversity Conference. There is a divergence of views amongst Parties as to whether or not the analysis is

[27] Available at: https://bch.cbd.int/database/record.shtml?documentid=114285 (2019), and https://bch.cbd.int/database/record.shtml?documentid=112053 (2017)

[28] Document CBD/SYNBIO/AHTEG/2019/1/3.

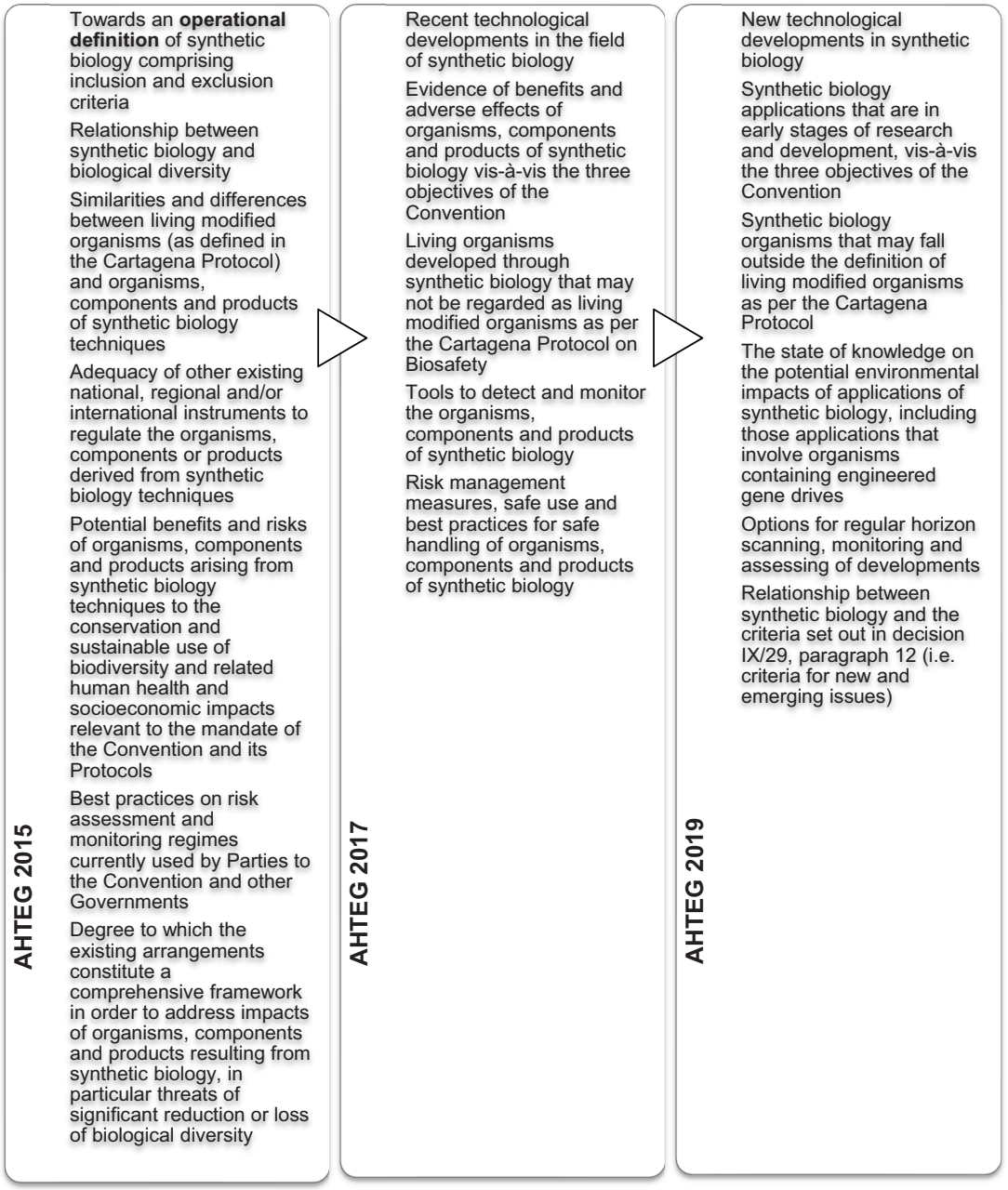

FIGURE 6 | Summary of issues addressed by the AHTEG on Synthetic Biology in 2015, 2017 and 2019.

actually needed to justify continued synthetic biology discussions under the CBD. Some Parties are resistant to completion of the formal analysis and of the view that the topic is clearly relevant to the CBD's objectives and of sufficient importance to be addressed under the CBD and its Protocols (e.g., see the 2019 NEI proposal by Norway[29]). Conversely, there are Parties of the view that in the absence of this completed analysis, the establishment, continuation and expansion of the CBD synthetic biology program of work cannot be justified, particularly when there are many other obligations parties have, and commitments they have agreed to that are aligned with the CBD's strategic plan, such as the Aichi Targets, that require extensive resources (e.g., see the 2019 submissions of information by Australia and Brazil[30]). At the 2020 Biodiversity Conference, CBD Parties are expected to adopt an ambitious new strategic plan, currently

[29] Available at: https://www.cbd.int/doc/emerging-issues/ntf-2019-041-submission-norway-en.pdf

[30] Available at: https://bch.cbd.int/synbio/submissions/

referred to as the Post-2020 Global Biodiversity Framework, which will also likely require implementation and evaluation of new targets at national levels[31].

The 2019 submissions of information[32], online discussions[33] and report of the AHTEG on Synthetic Biology[34] also demonstrate that a broad range of views exist on each of the NEI criteria, from these being satisfied by "synthetic biology" as a general concept (e.g., the 2019 NEI proposal by Norway, and the 2019 submissions of information by Finland and Malaysia[35]), or that specific applications such as gene drives (e.g., the 2019 submission of information by Bulgaria) and genome editing (e.g., the 2019 submission of information by Austria) qualify as NEI, to synthetic biology not meeting the NEI criteria (e.g., the 2019 submissions of information by Japan and New Zealand). There are also a range of views as to how the NEI criteria should be applied, e.g., how many of them need to be considered, their relative weighting, and how many of them need to be satisfied before something can be identified as a NEI.

Gene Drives

The topic of gene drives features prominently in synthetic biology CBD discussions, including any and all possible actual or conceptual applications, e.g., insects, mammals and plants (National Academies of Sciences Engineering and Medicine [NASEM], 2016b; Australian Academy of Science [AAS], 2017; Barrett et al., 2019), and types of drives, and is arguably one of the major drivers of the present-day CBD debate. It is also likely to feature in any deliberation on the NEI issue in the 2020 meetings. While there is no consensus that gene drives are "synthetic biology," there appears to be a general consensus that organisms containing engineered gene drives are LMOs within the scope of the Cartagena Protocol[36]. This means that organisms containing engineered gene drives are within the scope of LMO regulatory frameworks at the international level, and at regional and national levels of Parties that have implemented the Cartagena Protocol.

The COP14 decision of the CBD Parties in 2018 called upon Parties (and other governments) to "apply a precautionary approach" when considering introducing organisms containing engineered gene drives into the environment, with such decisions to be based on scientifically sound case-by-case risk assessments, and with risk management measures in place to avoid or minimize potential adverse effects (Decision XIV/19). In parallel, Cartagena Protocol Parties made a decision at COP-MOP9 in 2018 under the agenda item of LMO risk assessment and risk management (Articles 15, 16, and Annex III) recognizing that risk assessment guidance for organisms containing engineered gene drives may need to be developed to assist regulators (Decision IX/13). This gave rise to a parallel program of work under the Cartagena Protocol for determining whether or not there is a need to develop such guidance. The work on this topic began with submissions of information[37], studies commissioned by the Secretariat[38], and discussions of the open-ended online forum[39]. In March 2020 the AHTEG on Risk Assessment and Risk Management will meet to deliberate all of the information received and make recommendations for consideration by SBSTTA24[40], which will be the basis of a decision by the Parties at COP-MOP10.

The question of whether or not risk assessment guidance is necessary may seem innocuous, however historically this has been a controversial issue under the Cartagena Protocol, with criticism of the process for the development of guidance materials and the utility of its outcomes (Hokanson, 2019). This controversy led to the establishment of a formal process for the "identification and prioritization of specific issues of risk assessment of living modified organisms that may warrant consideration" (Decision IX/13 Annex I). The current program of work, in effect, is testing the process by applying a defined set of criteria to organisms containing engineered gene drives and to LM fish. The AHTEG will conduct an analysis and make a recommendation regarding whether or not there is a need to develop guidance, as well as recommendations on any adjustments that should be made to the criteria for prioritization of issues for risk assessment.

The submissions of information in 2019 on this topic[41] indicate that Parties and other governments have not yet received any applications for environmental release of organisms containing engineered gene drives, and hence there is limited direct experience in conducting risk assessment of such organisms. Some regulatory agencies are in the process of reviewing or have already reviewed their procedures for research with gene drive organisms in containment and acknowledge that the general principles and methodology for risk assessment and management, experience from LMO risk assessment, as well as knowledge from fields such as biocontrol agents and invasive alien species, will be relevant to performing risk assessment of organisms containing engineered gene drives. Challenges that are anticipated when performing environmental releases of such organisms are mainly related to the fact that the technology is targeting wild populations and may be irreversible, and thus the step-wise approach to environmental releases, as practiced with other types of LMOs, may require adaptation.

In regard to the NEI analysis for synthetic biology, the primary concerns that emerge specifically for organisms containing engineered gene drives in the 2019 report from the AHTEG on Synthetic Biology include a perceived lack of control and/or mitigation strategies, and traceability and/or detection tools once they are released into the environment, as well as their potential geographical spread and rate of spread. However, the report also hints at the need to consider such concerns in the broader context

[31] Zero draft document of 6 January 2020 CBD/WG2020/2/3.

[32] See: https://bch.cbd.int/synbio/submissions/

[33] See: https://bch.cbd.int/synbio/open-ended/discussion/

[34] Document CBD/SYNBIO/AHTEG/2019/1/3.

[35] Available at: https://bch.cbd.int/synbio/submissions/

[36] Document CBD/SYNBIO/AHTEG/2019/1/3.

[37] March 2019; available at: https://bch.cbd.int/onlineconferences/submissions.shtml

[38] Available at: http://bch.cbd.int/onlineconferences/studies.shtml

[39] January 2020; aee: http://bch.cbd.int/onlineconferences/forum_ra/discussion.shtml

[40] See: https://www.cbd.int/meetings/CP-RARM-AHTEG-2020-01

[41] Available at: http://bch.cbd.int/onlineconferences/submissions.shtml

and taking into account the potential benefits such as human well-being[42]. This consideration is especially relevant to gene drives given that the most advanced application, with field trial releases into the environment envisaged in the near term, is in mosquitoes for the control of malaria[43].

As noted above, the question of how to proceed with organisms containing engineered gene drives is contentious in other fora such as the IUCN [see section International Union for the Conservation of Nature (IUCN)], with a 2016 resolution calling for what was, in effect, a moratorium on gene drive research for conservation purposes[44]. In early 2020, the European Parliament voted on a resolution (European Parliament, 2020) calling on the EU Commission and the Member States to support a CBD COP15 decision for a global moratorium on releases of organisms containing engineered gene drives into the environment, including in experimental field trials.

Genome Editing

Another contentious synthetic biology topic that may be addressed by SBSTTA24 and COP15 is whether or not there are new synthetic biology developments that result in living organisms that are not within the scope of the Cartagena Protocol LMO definition (see **Figure 2**). The 2019 submissions of information, online discussions and the report of the AHTEG on Synthetic Biology indicate that this is a challenging question to address as it is subject to legal and technical interpretations, e.g., the content of the Cartagena Protocol definition of "modern biotechnology" (see **Figure 2**). It is also evident that it is subject to societal/community values, and how Parties apply the precautionary principle. Views differ most on this topic in regard to organisms developed via certain genome editing applications, as well as "transiently modified organisms," with relatively less attention paid to non-living "entities" such as protocells.

For living organisms developed using genome editing techniques, the same question has been or is currently being examined at regional and national levels, toward the aim of providing clarity regarding the scope of LMO/GMO regulation. A number of Cartagena Protocol parties and other governments have created exclusions for certain categories of genome editing technologies or products where these could have also been obtained through spontaneous processes or through the use of other (conventional) tools and methods (Dederer and Hamburger, 2019). Those countries have implemented such exclusions based on their implementation of the Cartagena Protocol definition of "modern biotechnology" whereby a "novel combination of genetic material" does not involve DNA changes that could have been obtained spontaneously or with the use of other methods. In these cases, the organism is managed in the same way as other non-LMO organisms. In the CBD synthetic biology discussions, these countries generally disagree that genome editing should be dealt with at the international level (e.g., the 2019 submission of information by Brazil), or that all applications of genome editing could be considered synthetic biology (e.g., the 2019 submissions of information by Australia and New Zealand), or that it requires special consideration within the Cartagena Protocol agenda item of LMO risk assessment and risk management (Articles 15 and 16, Annex III) (see SBSTTA Recommendation XXII/2). Conversely, there are participants in the CBD synthetic biology discussions that view such "non-LMO" organisms as a regulatory gap that needs to be addressed in this forum[45].

In regard to the NEI analysis for synthetic biology, one of the primary justifications for including genome editing appears to be a perceived lack of availability of detection methods for identification, particularly in regard to organisms developed using genome editing that have few DNA base changes and it may not be possible to distinguish them from other (non-edited) organisms (see the 2019 report of the AHTEG on Synthetic Biology[46]). Cartagena Protocol agenda items for COP-MOP10 that overlap with this discussion include LMO identification (Article 18) in the context of unintentional transboundary movements and emergency measures (Article 17). In 2019, the Online Network of Laboratories for the Detection and Identification of LMOs established under the Cartagena Protocol held online discussions to share their experience on the detection and identification of LMOs developed using genome editing and synthetic biology[47]. In those discussions it was evident that experience and/or technical capabilities are currently lacking in this area, but technologies are continually developing and these could be tested for feasibility.

Digital Sequence Information (DSI)

Digital sequence information (DSI) is an issue that arose from the CBD synthetic biology discussions, and since 2016 has been a substantial stand-alone agenda item under consideration jointly by the CBD and the Nagoya Protocol. It is expected to be a major topic at the upcoming Biodiversity Convention, where it will also be deliberated in the context of the Post-2020 Global Biodiversity Framework. DSI is a highly polarized issue that is currently under debate in several international fora, including the UN Law of the Sea Convention (UNCLOS), in relation to a new treaty under negotiation that includes marine genetic resources in areas beyond national jurisdiction; the International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA) in relation to plant genetic resources; and it is under evaluation by the World Health Organization in relation to the Pandemic Influenza Preparedness Framework (Manheim, 2016).

Under the CBD, the origin of the DSI issue can be ascertained from the report of the 2015 meeting of the AHTEG on Synthetic

[42] Document CBD/SYNBIO/AHTEG/2019/1/3.

[43] See: https://genedrivenetwork.org/open-letter/

[44] Resolution WCC-2016-Res-086: Development of IUCN Policy on Biodiversity Conservation and Synthetic Biology. Available at: https://portals.iucn.org/library/node/46503

[45] E.g., see the online discussion "Topic 5: Consider whether any living organism developed thus far through new developments in synthetic biology fall outside the definition of living modified organisms as per the Cartagena Protocol," held 18-24 March 2019. Available at: https://bch.cbd.int/synbio/open-ended/discussion/?threadid=9602

[46] Document CBD/SYNBIO/AHTEG/2019/1/3.

[47] See: https://bch.cbd.int/onlineconferences/portal_detection/2019discussions.shtml

Biology[48], where the "potential adverse effects" of synthetic biology were considered. One such effect listed in that report is the obtaining of benefits from the use of DNA information obtained from a genetic resource without fair and equitable benefit sharing, which is a CBD objective (see **Figure 1**). The CBD/Nagoya Protocol access and benefit sharing (ABS) regime applies to "users" and "providers" of "genetic resources," with "genetic resources" generally understood to constitute physical material, such as cell or tissue samples from an organism. A perceived feature of synthetic biology is increasing use and exchange of DNA sequence information without the need for each user of that information to access the source physical resource to which CBD/Nagoya Protocol ABS obligations apply (e.g., subject to prior informed consent and mutually agreed terms; CBD Article 15), resulting in a form of "misappropriation" of that genetic resource and bypassing of the provisions of the Nagoya Protocol. The report from the 2015 meeting of the AHTEG on Synthetic Biology recommended that the Nagoya Protocol COP-MOP "set up mechanisms" for clarifying this issue as it relates to ABS.

Additional commentary in the report of the 2015 meeting of the AHTEG on Synthetic Biology points to a "shift in the understanding of what constitutes a genetic resource," and this lies at the heart of the continuing CBD/Nagoya Protocol debate. Views on this are highly polarized, with some Parties of the view that the definition of "genetic resources" can only refer to tangible material and not intangible information, whereas other Parties strongly believe that information is within the scope of "genetic resource," particularly those Parties that view themselves predominantly as "providers" rather than "users" of genetic resources. This user/provider dichotomy is also evident in the DSI debates under the ITPGRFA and in the development of the new treaty under UNCLOS.

In 2016, the CBD (COP13; Decision XIII/16) and Nagoya Protocol (COP-MOP2; Decision II/14) Parties jointly decided to establish a program of work on DSI which included submissions of information, a commissioned study, and an AHTEG on Digital Sequence Information, with the outcomes of that work to be considered by SBSTTA. "DSI" is itself another undefined term, and this first program of work was focused on examining terminology and different types of DSI, and its relationship with the objectives of the CBD and Nagoya Protocol. While the initial discussions on DSI appeared to apply specifically to electronic DNA sequence information, the 2018 report of the AHTEG on Digital Sequence Information contains a broad list of information "relevant to the utilization of genetic resources"[49]. This ranged from genetic and biochemical information that may be obtained from a (physical) genetic resource, to "observational" information associated with it, e.g., ecological relationships, taxonomy, phenotype.

The outcomes of the first DSI program of work were extensively debated at the 2018 meeting of SBSTTA22, as evident by the heavily bracketed text in the resulting recommendation that reflected the lack of consensus amongst the Parties (Recommendation XXII/1). This recommendation was the basis for further contentious debate at COP14/COP-MOP3 later that same year. The eventual decision of COP14 recognizes the divergence of views amongst Parties, and sets out a "science- and policy-based process" aimed at assisting the Parties to work to resolve this (Decision XIV/20). The process includes further submissions of information[50], four commissioned studies, and extension of the AHTEG on Digital Sequence Information. The topics to be examined in the program of work were aimed at improving "conceptual clarity," including: terminology and scope, traceability and use of public databases, benefit sharing arrangements for commercial and non-commercial (i.e., research) uses of DSI, and how DSI is considered within existing domestic ABS measures. The COP14 and COP-MOP3 decisions (Decision III/12) refer the outcomes of the work of the AHTEG on Digital Sequence Information to the Open-Ended Working Group on the Post-2020 Global Biodiversity Framework, rather than SBSTTA, who are to submit the outcomes of their deliberations to COP15/COP-MOP4 in October 2020.

At the time of writing, the program of work is in progress, with drafts of the four commissioned studies released in late 2019 for "peer review"[51], and the AHTEG on Digital Sequence Information scheduled to meet in March 2020. To date there has been limited discussion on DSI in the context of the Post-2020 Global Biodiversity Framework, with the draft text of this released in January 2020 referring to ongoing work in this area[52].

CONCLUSION

In this paper we have provided an overview of the major developments in biotechnology regulation since the first discussions on this topic at the 1975 Asilomar conference on recombinant DNA. While the technologies and the range of organisms developed have evolved since then, accompanied by accumulated experience and expertise in assessing and managing risks, the CBD synthetic biology discussions are, in essence, based on the same concerns about safety and appropriate regulatory oversight that brought about the Asilomar conference. These concerns are at the heart of most of the synthetic biology-related discussions that are anticipated at the 2020 Biodiversity Conference, and these are further conflated with broader political and societal issues. Collectively, these have contributed to the ever-expanding CBD synthetic biology work program, the evidence-based NEI analysis remaining incomplete for almost 10 years, and the relatively new dimension of access and benefit sharing in relation to information.

In the view of the authors, the CBD discussions on synthetic biology can be seen as an exceptionally prolonged version of

[48] Document CBD/SYNBIO/AHTEG/2015/1/3.

[49] Document CBD/DSI/AHTEG/2018/1/4.

[50] Available at: https://www.cbd.int/dsi-gr/2019-2020/submissions/

[51] Available at: https://www.cbd.int/dsi-gr/2019-2020/studies/

[52] Document CBD/WG2020/2/3.

the Asilomar conference. However, an important distinguishing feature is that the CBD discussions are relatively lacking in participation by its practitioners. This is possibly due to the complex and resource-intensive nature of CBD processes, and the fact that these are Party (or government)-led processes and the scientific community can only "observe" unless they are directly engaged by governments. While there are members of the scientific community that contribute to the CBD discussions, stronger involvement is essential to support evidence-based decision-making and the development and/or adjustment of effective, adaptive and proportionate regulation. The optimism and excitement of the scientific community for providing solutions to global challenges with synthetic biology stands in stark contrast to the CBD debates, which have spent little time on acknowledging the demonstrated or supporting the potential contribution of biotechnology toward the achievement of the biodiversity and sustainability objectives at the heart of the CBD.

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FK and AA conceived and designed the review. FK drafted the manuscript with support from AA who critically reviewed it and also contributed content.

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Argentina's Local Crop Biotechnology Developments: Why Have They Not Reached the Market Yet?

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Plant biotechnology in Argentina started at the end of the 1980s, leading to the development of numerous research groups in public institutions and, a decade later, to some local private initiatives. The numerous scientific and technological capacities existing in the country allowed the early constitution in 1991 of a sound genetically modified organisms biosafety regulatory system. The first commercial approvals began in 1996, and to date, 59 events have obtained permits to be placed on the market, however, only two have been developed locally by public-private partnerships. The transgenic events developed at public institutions pursue different objectives in diverse crops. However, once these events have been developed in laboratories, it is difficult to move toward a possible commercial approval. In this work, we analyze several reasons that could explain why local developments have not reached approvals for commercialization, highlighting aspects related to the lack of strategic vision in the institutions to focus resources on projects to develop biotechnological products. Although progress has been made in generating regulatory rules adapted to research institutes (such as the regulations for biosafety greenhouses and ways of presenting applications), researchers still do not conceive regulatory science as a discipline. They generally prefer not to be involved in the design of regulatory field trials or regulatory issues related to the evaluation of events. In that sense, some of the aspects considered a regulatory affairs platform for the public scientific system and the reinforcement of laboratories that perform tests required under the Argentine regulation.

Keywords: regulatory system, GMO biosafety, Argentine regulation, local developments, commercial approval

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BRIEF HISTORY OF BIOTECHNOLOGY IN ARGENTINA

Plant biotechnology in Argentina started at the end of the 1980s, leading to the development of numerous research groups in public institutions and, a decade later, to some local private initiatives. However, a prospective analysis of the local capacities of Argentina for the development and marketing of events derived from biotechnology would have led to a much more optimistic scenario than the one observed nowadays. Argentina's experience with plant biotechnology began with pioneers such as Esteban Hopp, at the National Institute of Agricultural Technology (INTA), and Alejandro Mentaberry, at the National Scientific and Technical Research Council

(CONICET), in the late 1980s, both of whom mentored the subsequent generations of specialized academics in the area.

Farmers in Argentina have always rapidly adopted new developments and technologies. Indeed, Argentine fields currently have more than 24.9 million hectares of GM crops, 19.2 of which are of soybean (almost 100%), 5.5 of maize (96%), and 0.3 of cotton (almost 100%). These data indicate that farmers are not reluctant to adopt these crops, the vast majority of which are developed abroad (ISAAA, 2018).

In 1991, the National Advisory Commission on Agricultural Biotechnology (known by the Spanish acronym, CONABIA), whose function consists of reviewing the safety assessments of biotechnology events, was formed. CONABIA is still operative today[1] and its members include, among others, specialists in the fields of genetics, plant physiology, and agronomy. A significant aspect of the Argentine regulatory system is that it is widely recognized as being a structure that has remained "uncontaminated" by bureaucratic history, where scientific and technical credibility and enforceability prevail, which is critical when dealing with a sensitive issue for society, such as GM crops (Vicién and Trigo, 2017).

Since its creation, CONABIA has been instrumental to the successful evaluation of more than 50 different (single and stacked) events. Thanks to its outstanding academic members and excellent track record in the field, in 2014, CONABIA was recognized as a Reference Center for the Biosafety of genetically modified organisms (GMOs) by the FAO. Considering these facts, Argentina should have been much more successful in the deregulation of its local biotechnological events. However, only two out of the more than fifty events that have been approved for commercialization were developed locally.

The regulatory process in Argentina is established in Resolution 763/2011 issued by the Ministry of Agriculture. This Resolution establishes a procedure divided into three steps: (i) an environmental assessment performed by CONABIA, (ii) a food and feed safety evaluation performed by the National Agri-Food Health and Quality Service (known as SENASA, by its Spanish acronym), and (iii) an evaluation of its impact on the agricultural market. Once each step is completed, a Decision Document is drafted, which must be favorable for the event to be approved. The procedure is the same for both local and imported events. Most of the events that have passed through the regulatory process have been developed by private companies, mostly from the Northern Hemisphere[2] (**Figure 1**: events approved in Argentina).

Table 1 shows the two national events approved for commercialization that have completed all the steps of the regulatory process: the abiotic stress-resistant and herbicide-tolerant Soybean HB4, developed by the local business company INDEAR (a public and private partnership formed by CONICET and the enterprise BIOCERES), and the virus Y-resistant Potato PVY, developed by CONICET and achieved by the company Sidus-Tecnoplant, a public-private collaboration.

In 2015, another event was almost approved for commercialization: sugarcane with glyphosate resistance. This event passed the environmental and food and feed safety assessments, but failed to pass the agricultural market impact evaluation, possibly because of the negative public perception of sugarcane stakeholders[3]. The developments of sugarcane varieties are carried out by both public and private sector institutions.

Wheat HB4, another local development by INDEAR, which has drought resistance, is currently undergoing the evaluation and has already achieved approval from both CONABIA and SENASA, but it is still awaiting the final decision from the Agricultural Markets Office[4]. In that sense, beyond regulations, another aspect of the Argentine system that has to be considered is the internalization of potential trade problems, which is based on its position as a net exporting country. This is generally one of the main causes for the delay in approvals, since the government weighs the consequences of any new products on the Argentinian market (Vicién, 2012).

Regarding the remaining local developments listed in **Table 1** (a list that may not be exhaustive), many have not gone beyond the laboratory step, others have only completed the greenhouse step, and very few have been evaluated in the field. This situation inevitably raises questions regarding the difficulties faced by local developments when looking for deregulation.

Other countries in the region are in similar situations regarding the adoption of biotechnology and are working to establish their deregulation procedures. FAO asked CONABIA to assist other countries that were establishing their regulatory frameworks, a program that is proving successful in training specialists on how to perform risk assessment for GM crops. While Argentina's regulatory system serves as a reference for many countries, it is important to note that the system does not appear to support local developments. Fortunately, there are exceptions in the region, such as the virus-resistant beans obtained by the Brazilian Agricultural Research Corporation (EMBRAPA by its acronym in Portuguese), developed by Francisco de Aragao's Group (Faria et al., 2016). So far, this has been the only case in which an entirely public development achieved approval by the Brazilian National Technical Biosafety Commission (CTNBIO by its acronym in Portuguese), having also completed every assay required in publicly funded labs[5].

Over the last 30 years, the scientific-technological system in Argentina, composed of public institutions and universities, has led to the development of many GM crop events, such as potato, alfalfa, wheat, maize, sunflower, sugarcane, soybean, lettuce, and cotton (**Table 1**). These were achieved mostly through funding from institutions such as INTA, CONICET, Universities, and the National Agency for Scientific-Technical Promotion (ANPCyT by its acronym in Spanish) and other public sources. The projects are financially and economically evaluated taking into account research and development costs, regulatory aspects (biosecurity

[1]https://www.argentina.gob.ar/convocatoria-conabia

[2]https://www.argentina.gob.ar/ogm-comerciales

[3]http://www.sitioandino.com.ar/n/176588-ingenios-se-resisten-a-la-cana-de-azucar-transgenica/

[4]https://www.clarin.com/economia/economia/gen-resistencia-sequia-divide-aguas-gabinete-macri.html

[5]http://ctnbio.mctic.gov.br/documents/566529/686210/Parecer+Consolidado+Francisco+Zerbine.pdf/2217911a-07fb-4643-8757-e9badcbe5516?version=1.0

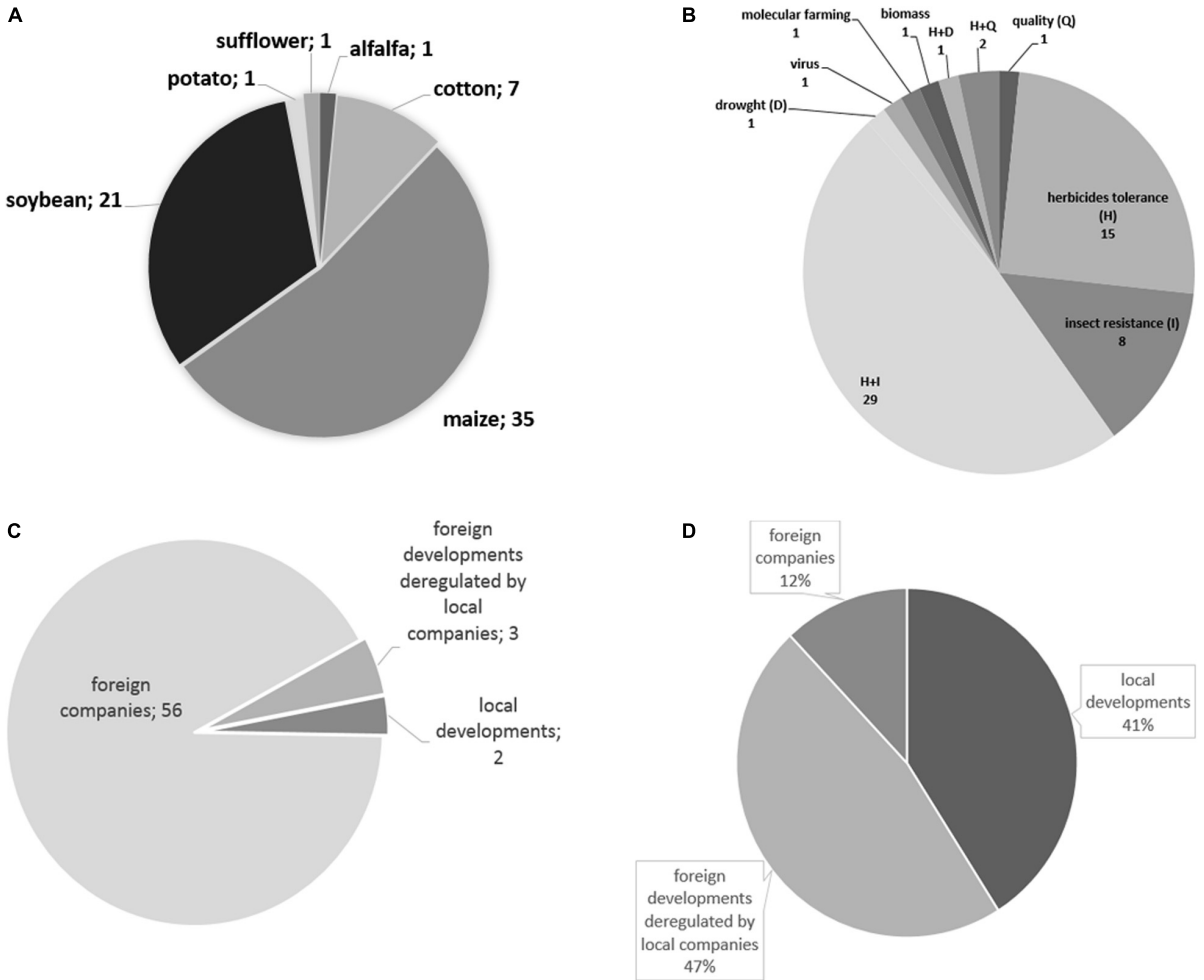

FIGURE 1 | Approved biotechnological events in Argentina. Graphics made based on data published on the site https://www.argentina.gob.ar/ogm-comerciales. **(A)** number of events by crop; **(B)** number of events by trait; **(C)** number of events approved by type of developer; **(D)** cases of gene editing consulted (A. Whelan, personal communication).

and varietal registration), potential benefits for farmers and for the value chain, prospective on possible markets (both internal and external), and relative sizes and possible degree of adoption. After obtaining the desired prototypes for each laboratory event, developments only reach the stage of growth chamber or greenhouse trials. However, once they are stabilized and multiplied, in many cases, there is not enough funding to advance to field trials and complete the remaining steps of the regulatory procedure.

NEW NORMS FOR NEW TECHNIQUES

The regulatory framework has been recently updated in Argentina. The country pioneered the development of regulations for the so-called "new breeding techniques" (NBTs), as specified in Resolution No. 173 of 2015 from the Ministry of Agriculture (Whelan and Lema, 2015) and also present in the updated version from the same Ministry (Resolution No. 36, 2019). This Resolution states that, to be considered as a "Genetically Modified Organism," the product must possess a novel combination of genetic material obtained through the use of modern biotechnology in accordance with the definition from the Cartagena Protocol on Biosafety (Secretariat of the Convention on Biological Diversity [SCBD], 2000). Under these guidelines, the applicants must describe in detail the intended modifications and the way they plan to obtain them in an "Instance of Prior Consultation" (ICP by its Spanish acronym). CONABIA will send an answer to the applicant within 60 days to determine the regulatory status of the product. This procedure allows researchers to know whether their product will be considered as a GMO or not, even before starting laboratory work. The procedure has served as an incentive for private companies and public institutions to undertake new projects, with knowledge at the outset that costly regulatory testing will not be necessary. To date, most of the inquiries received by the authorities have come from locally developed products (Lema, 2019).

TABLE 1 | List of biotechnological developments in Argentina.

Crop	Trait	Maximum degree of progress achieved				Type of institution
		Greenhouse/ laboratory	Field trials	CONABIA/SENASA approved	Commercial approved/deregulated	
Potato	Bacteria	×				Public
	Fungal	×				
	Quality	×				
	Nutrition facts	×				
	Virus PVX-PLRV	×	×			
	Virus PVY	×	×	×	×	Public/private
Wheat	Nutrition facts	×				Public
	Abiotic stress	×	×			
	Abiotic stress	×	×	×		Private
Alfalfa	Herbicide	×	×			Public
	Abiotic stress	×	×			
Orange	Virus	×	×			Public
Maize	Abiotic stress	×	×			Public
	Abiotic stress	×	×			Private
Cotton	Boll weavil	×				Public
Sugarcane	Virus	×	×			Private
	Herbicide	×	×	×		
Peach	Quality	×				Public
Tomato	Abiotic stress	×				Public
	Leaf area	×				
	Nutrition fatcts	×				
Lettuce	Virus	×				Public
	Fungal	×				
Sunflower	Fungal	×				Public
Grape	Abiotic and abiotic stress	×				Public
Soybean	Herbicide	×	×	×	×	Public/private
	Abiotic stress	×	×	×	×	
Paspalum	Abiotic stress	×				Public
Chloris gayana	Abiotic stress	×				Public
Fescue	Herbicide	×	×			Public

Gene editing, one of the NBTs discussed above, is one promising new biotechnological approach to improve crops that is considered more precise and can avoid the insertion of unnecessary genes. Crops modified using gene editing may be more easily adopted because products require a simpler regulatory procedure (Jones, 2015; Georges and Ray, 2017). The procedure described in Resolution No. 173/15 is streamlined for products derived from gene editing but it would still be subject to regulation, given that any product derived from the application of biotechnology is still regulated until it is determined that it does not contain stable DNA insertions. Before then, all associated material must be handled in contained and confined conditions.

DIALOGUE BETWEEN RESEARCHERS AND PUBLIC REGULATORS IN ARGENTINA

In 2014, given difficulties faced by developers in Argentina, REDBIO (a non-profit organization bringing together plant biotechnology labs across Argentina) held two workshops to debate and share ideas regarding the prevailing situation. In that sense, two official statements were drafted and addressed to the authorities responsible for regulation at the Ministry of Agriculture.

In these workshops, the various problems faced by researchers regarding the current regulatory system in Argentina were considered. The conclusions are listed below.

Regarding Policies

- General lack of State support for most research information and commercialization of GM events.
- Insufficient sources of resources and State funding to go through the trials required for deregulation.
- The non-existence of formal State structures to facilitate, organize, and present regulatory data to the relevant agencies.

Regarding the Regulatory Process

For Developers

- They are often unaware of the general regulatory process.
- No guidelines specify how developers should start with the regulatory process.
- The processes and steps to complete, as well as the initial data required, are unclear.
- Many developers are not up to date on the resolutions setting out the process.

For the Authorities

- There is no coordination between the agencies and offices involved in the three steps of assessment.
- There is an overlapping of the information required from each agency.
- Requirements and criteria are often excessive.

Regulatory Data Generation

- There is a need for a complete diagnosis of the available infrastructure and capabilities of the national science and technology system.
- There is insufficient funding for regulatory studies.
- There is insufficient information and training on the steps to follow in the regulatory process.
- There is a need for a definition of national and international harmonized quality standards in confined field trials.

It has to be highlighted that researchers in public institutions such as INTA have always worked collaboratively with breeders, who have extensive experience in intellectual property issues, varietal registration, and the respective procedures established by the National Institute of Seeds (INASE by its acronym in Spanish).

To respond to the needs raised in the 2014 workshops, two trainings for researchers were organized by public institutions such as INTA and CONICET and NGOs such as Redbio, ILSI, and Argenbio. Researchers from all plant biotechnology development centers of Argentina participated in them and their projects were analyzed concerning how regulatory studies should be addressed.

The authorities of the Ministry of Agriculture and the Ministry of Science and Technology who participated in these workshops and acknowledged the problems discussed there created a competitive financial funding program for regulatory studies. This program was organized by ANPCyT in 2015 and was called FONREBIO (Biotechnological Products' Regulation Funding)[6]. This funding was initially conceived as a non-reimbursable subsidy for public institutions. However, by the time of its issuance and implementation at the end of 2015, it became a sort of reimbursable loan, which required the approval for a private source of partial backing or commitment for quick insertion into the market. It was announced as a loan of ARS 20 million (USD 1 million at that time), consisting of up to 80% of the project's total cost, with at least 20% of its funding coming from private sources. This restricted the chances of projects coming from public institutions and forced these institutions to seek support from companies interested in investing in the projects. Furthermore, the chances for a project to advance were tied to its intrinsic potential for commercial success.

Similarly, INTA established an internal contest for the use of royalty funds owned by the institution (known as "*Fondos de valorización tecnológica*"). The funding was meant to promote the insertion of products developed by INTA, including biotech events, into the market. To apply, the development must be commercially viable and easily adopted by producers, which again limits the availability of funding to economically competitive developments only. At the beginning of 2018, the funding consisted of ARS 20 million (USD 1 million back then but only USD 300 thousand nowadays due to the devaluation of the Argentine peso) directed at projects of many different origins, including biotech events. The amount offered quickly proved to be insufficient. Once again, an initiative to boost local developments ended up being inadequate to achieve that goal.

As previously mentioned, local projects can have many different goals. Some of them focus on productivity, which gives them better chances to compete for funding to pay for regulatory studies successfully, whereas others are meant to enhance quality, or are directed at small producers or family farms, which leaves their chances to acquire private backing limited or completely cut off. This means that they end up being unable to pay for the cost of the regulatory process, thus keeping the product from ever reaching the market.

Regarding the costs of the deregulation process, some estimates indicate that it is roughly ten times that of the development itself. These estimates include the costs of gene discovery together with those of the deregulation in various countries, which is partly why the total amount needed varies so much (Kent, 2004; Falck-Zepeda et al., 2012; Prado et al., 2014). Variation in cost depends on the number of countries where it is filed and the nature of the studies required according to the approval policies of each country. Even though in most cases the necessary studies to achieve approval for commercialization are well established, the amount of money needed to complete them is up for debate, and can be high enough for small businesses or public institutions to abandon or delay the marketing of locally potentially valuable products. Currently, mainly private multinational companies can afford the regulatory burden of approvals in different countries.

Another factor that makes this process even more expensive is the quality certification needed for the data obtained in the regulatory studies. For the EU, the data required must be GLP (Good Laboratory Practices) certified. This certification is handled by the Argentine Accreditation Body (OAA by its Spanish acronym). So far, only a few institutions have been able to achieve this certification, which is expensive. SENASA and CONABIA do not currently require this certification but procedures and data integrity are thoroughly examined.

The current state of event approval across the globe must also be taken into consideration. To avoid problems with imports and exports, the approval of a single event is often requested in more than one country at the same time (for example, a maize

[6]http://www.mincyt.gob.ar/convocatoria/fonrebio-11901

event may be presented simultaneously in the United States, European Union, Brazil, Argentina, Colombia, and Paraguay). Every country added to that list results in additional costs and resources. To facilitate the process for their products, many international companies engage their own Regulatory Affairs Departments. This practice allows an efficient organization of both human and financial resources. Until now, there is nothing similar in public institutions.

Based on all the above, the following questions can be posed: Can a local project achieve deregulation in the country? Can it achieve the same acceptable standards for safety as a previously approved privately funded one? Can locally funded projects afford de-regulation in other countries?

THE PARADOX

As a result of the foregoing, public institutions find it almost impossible to raise funds for the deregulation process, the cost of which exceeds any funding that may be obtained. Thus, a paradox is established, whereby products derived from plant biotechnology, such as GMOs, are developed to address production problems and improve crop quality in ways that conventional breeding cannot achieve, and yet, for these products to be approved and thus be used by farmers, they must meet the criteria set by regulatory authorities in each country where they are expected to be commercialized. The required regulatory studies are extraordinarily expensive and can only be paid for if the institution or company where the product was developed has the necessary funding.

Consequently, only private developments with sufficient funding and adequate resource management are able to pay for the required studies and their certification. Therefore, the only products to ever reach the market are those derived from private initiatives of transnational firms. Developments seeking to solve problems for local production or small farmers are seldom given a chance to reach approval, as they are less attractive for large companies because markets and sales are smaller in size. Furthermore, even when they are granted approval, after much effort and search for company help, as it happened with PVY-resistant potato, developers may find obstacles to commercialization because of public perception barriers. Another controversial case is that of wheat HB4®, which was approved by two out of three agencies (CONABIA and SENASA) but rejected by the agricultural market evaluation, because of the unfavorable public perception of the crop's value chain.

In this context, further questions that can be posed include: How can this problem be solved? Which institutional paths can be explored? What should be proposed?

As mentioned before, private multinational companies have regulatory affairs departments, composed of professionals in charge of designing the studies, managing agency permits, performing field trials, and conducting follow-up of developments to comply with regulatory criteria all over the world. Public institutions do not have anything like that. A good start for public policies meant to help the development of local biotechnology through the organization of human and financial resources would be the creation of a government agency for GMO regulatory affairs as a shared platform to make the process faster, easier, and more efficient. Local researchers and developers must also consider starting a dialogue with every participant in the value chain, including producers and coordinators of crop breeding programs, from the very beginning of the initial development (the original idea). These considerations can be applied to GMOs, as well as to products obtained through the application of gene editing and other new tools of biotechnology.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://www.argentina.gob.ar/agricultura/alimentos-y-bioeconomia/ogm-comerciales.

AUTHOR CONTRIBUTIONS

Both authors participated in the drafting of this manuscript as individual experts in their fields, and are solely responsible for the contents. Any views expressed in this manuscript are the views of the authors and do not necessarily represent the views of any organization, institution, or government to which they are affiliated or employed.

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Gene Editing Regulation and Innovation Economics

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Argentina was the first country that enacted regulatory criteria to assess if organisms resulting from new breeding techniques (NBTs) are to be regarded as genetically modified organisms (GMOs) or not. The country has now accumulated 4 year of experience applying such criteria, reaching a considerable number of cases, composed mostly of gene-edited plants, animals, and microorganisms of agricultural use. This article explores the effects on economic innovation of such regulatory experience. This is done by comparing the cases of products derived from gene editing and other NBTs that have been presented to the regulatory system, against the cases of GMOs that have been deregulated in the country. Albeit preliminary, this analysis suggests that products from gene editing will have different profiles and market release rates compared with the first wave of products from the so called "modern biotechnology." Gene editing products seems to follow a much faster development rate from bench to market. Such development is driven by a more diverse group of developers, and led mostly by small and medium enterprises (SMEs) and public research institutions. In addition, product profiles are also more diversified in terms of traits and organisms. The inferences of these findings for the agricultural and biotechnology sectors, particularly in developing countries, are discussed.

Keywords: gene editing, innovation economy, biotechnology regulation, bioeconomy, genome editing, CRISPR-CAS, new breeding techniques, biotechnology indicators

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INTRODUCTION

The Argentine regulatory system for modern biotechnology applied to agriculture is recognized worldwide for being among the most experienced ones (Vicien and Trigo, 2017). Being one of the leaders in this field, in 2015 the country enacted a pioneer regulation for products of the so-called "new breeding techniques" (NBTs), including gene (or genome) editing. As described in Whelan and Lema (2015), products derived from NBTs are submitted to a case-by-case analysis in order to establish if they are genetically modified organisms (GMOs) or not. Such criteria also include cooperative links between the regulatory frameworks for GMOs and for conventional products, in order to avoid any safety or legal gap.

Technical details pertaining to scientific and legal regulatory criteria applied in this regulation can be found elsewhere, both in our recent publications (Lema, 2019; Whelan and Lema, 2019) and the updated regulatory texts (Infoleg, 2019a,b). There is also literature available that contextualizes this regulatory approach at the international level (Duensing et al., 2018; Eriksson et al., 2019; Metje-Sprink et al., 2020).

The study presented here further explores the implications for economic innovation of such regulatory activity in Argentina, by analyzing the profile of traits and organisms modified by NBTs that have been presented to the regulatory system. Although there is plenty of literature available about the impacts of GMO cultivation in Argentina and elsewhere (Brookes and Barfoot, 2018a,b, and references therein), it is not the same case for products derived from gene editing. Therefore, as we and others have discussed previously (Whelan and Lema, 2017; Maaß et al., 2019), from a policymaking perspective there is a need for studies pertaining to the potential socioeconomic impacts of gene editing applied to agriculture, including any modulatory effect that regulatory approaches can have on such impacts.

THE ROLE OF REGULATION IN INNOVATION PROCESSES

"Regulation" understood as the laws, norms and rules that order an economic, social or institutional process is essential to guide the technological development of countries, among other factors that also affect innovation processes. In a productive sector based on biological processes, such as the agroindustry sector, regulation is a tool that should be used to preserve the "welfare," in the broadest sense, of society as it adopts innovations. In other words, the enactment and application of regulations is part of policymaking, where the aim is to establish frameworks for safe and adequate development within the innovation system.

As a source of codified knowledge, regulations have a direct impact on technology diffusion because they affect the generation of new technologies, as well as decisions on their adoption by potential users (OECD, 1996; Geroski, 2000). In regards to technology development, regulations have similar properties to those of a "public good"; in that the main characteristics should be "openness" and "credibility." "Openness" refers to the situation of a regulation being accessible and applicable to all competitors, which is particularly important for small innovative companies because it grants certainty for market access. In addition, "credibility," refers to the State being able to create confidence that a norm is of general use (Temple, 2005).

Regarding the effect on potential adopters (i.e., developers and users) of a technology, the establishment of a regulation reduces uncertainty about technological characteristics by increasing the availability of information. Therefore, it facilitates their decision process (Kat and Oomen, 2007) and the diffusion of innovation. The combination of these effects on supply (technology developers) and demand (potential users of technology) makes regulations a key issue in any country's strategy for economic development.

The unnegotiable objective of establishing sanitary and phytosanitary regulations must be safety. Having said that, when different regulatory options provide an adequate level of safety, careful consideration should be given to select the option that is more likely to foster technological development, and thus avoid unnecessary brakes on the process of technological change (Ponte and Gibbon, 2005; Mancini, 2013; Tran et al., 2013).

According to a report by Moya-Angeler (2014), an increase in regulatory requirements usually hampers innovation by small enterprises, thus decreasing market competition, and ultimately driving a market concentration in large multinational companies (MNCs). This is particularly evident for regulations requiring extensive and expensive tests prior to the approval of a product, which discourage small and new innovative companies while granting a relative advantage to larger and established companies because they are better able to cope with the burden that this implies (Ashford and Heaton, 1983).

In this context, one of the current issues in development of agricultural biotechnology is analyzed next: the impact of regulatory requirements on innovations based on gene editing and other NBTs. An analysis of the Argentine experience may allow some conclusions to be drawn regarding the potential impacts on the agriculture and the biotechnology sectors. This would be a timely and valuable contribution to technology developers and policy makers in this area, as well as to the academic community working on "science and technology studies" (STS) (Hackett et al., 2008) particularly in the field of innovation economics.

COMPARATIVE ANALYSIS OF GMOs vs. GENE-EDITED PRODUCTS PRESENTED TO THE REGULATORY SYSTEM

Timeline

Figure 1 exhibits the timeline of GMO approvals in Argentina *vis a vis* the determinations of conventional or GMO status for products obtained using different NBTs. It should be noted that the term "product" in this study is used for referring to cases where a regulatory determination has been made on an organism, and not necessarily refers to products that are actually available on the market.

It is important to note that the situation of a GMO being authorized is comparable with a determination that an NBT

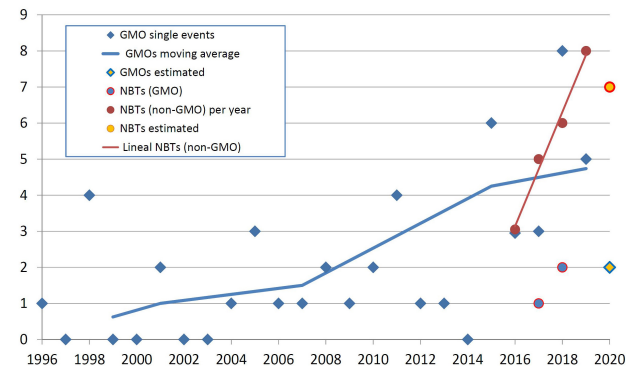

FIGURE 1 | The timeline of GMO approvals in Argentina and the determination of conventional or GMO status for products obtained using different NBTs. The horizontal axis represents the year of the regulatory decision, and the vertical axis represents the number of products. See text for details.

product is not a GMO. Both situations place the biotech crop at an equivalent instance, i.e., one step away from actual freedom to commercialize (that step being the registering of the product with the regulator of conventional products). Detailed comparison between the regulations for GMOs and conventional products including those obtained with NBTs in Argentina is provided in Whelan and Lema (2019).

In **Figure 1**, blue diamonds represent the number of new GMO single transformation events authorized per year since the first deregulation of an herbicide-tolerant soybean in 1996. The blue line is a moving average calculated on the basis of the period of Argentinean presidential terms of office (Wikipedia, 2020); this representation was included in order to help analyzing if there is a trend in the noisy data and, at the same time, to explore if there have been changes in public policy that might have influenced that trend. Finally, the yellow diamond for the year 2020 is an estimate based on the amount of GMO dossiers that have been filed recently and are currently under assessment.

Looking at NBTs in **Figure 1**, circles fully colored in red represent the number of NBT products that have obtained a determination of being non-GMO (i.e., conventional) organisms. The red line is a linear regression of such data; it was included to allow comparing with the changing slope of GMO approvals. The yellow circle for the year 2020 represents an estimate based on the number of informal inquiries that were attended recently.

Blue circles represent a few NBT products that were established to be GMOs; therefore, they should go through the GMO deregulation process, which would take several years for a subsequent approval. These cases were not considered further for the analyses presented next. For this reason, "NBT product" shall be understood as "non-GMO NBT product" for the remainder of this article.

Genetically modified organism approvals exhibit a trajectory that increases "noisily" but steadily. The noise at the yearly level is likely a consequence of assessing a time series made of small numbers that are the sum of few cases each year, and therefore it may be quite sensitive to particularities of individual cases. However, the moving average is always increasing, and it does not seem to be significantly affected by putative changes in biotech policies from one administration to the next. This average is likely growing in correlation with the generalized increase of traditional biotechnology development indicators, such as scientific publications, patents or R&D investment (Banerjee et al., 2000; Arundel, 2003; Reiss and Dominguez-Lacasa, 2016; OECD, 2019b).

In regards to NBTs, any insight from the very limited number of observations available shall be deemed preliminary. Having said that, it seems that NBT products, currently in the founding years, are emerging much faster compared with the foundational (or any other) period of GMOs. Roughly speaking, both product categories can be considered even now in terms of quantity of products arising per year, but if the apparent trends continue, NBTs will be significantly superior by numbers in the near future.

Although the same kind of comparison of relative development rates could have been made with the traditional indicators mentioned earlier, this measurement of "deregulation rate" is also enlightening, and perhaps even more useful to anticipate the actual use of these technologies in the field. This is because a comparison at the final stages of deregulation is obviously much closer to the actual market release compared with traditional indicators based on earlier stages of product development. Moreover, indicators based on advanced instances of deregulation are less likely to be skewed by proof-of-concept cases that ultimately were not destined to raise commercial interest.

Developer Profiles

Figures 2, **3** shows groupings of the cases introduced in **Figure 1** according to the developer's profile. The criterion used to identify a MNC is taken from Dunning and Lundan (2008), while small and medium enterprises (SMEs) were classified as such according to internationally recognized criteria (OECD, 2019a,c). All foreign MNCs in this study have headquarters in developed countries. All Argentine companies in this study are SMEs with no subsidiaries, except for one multinational seed company with headquarters in Argentina (present in just 6 countries and quite small compared to the foreign MNCs).

Figure 2 shows that GMOs are deregulated mostly by MNCs, and actually such developers were the only group throughout the first two decades of the regulatory system. Only during the last

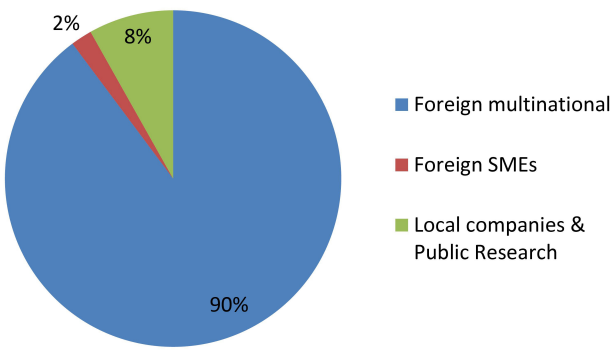

FIGURE 2 | GMO products by developer profiles. See text for details.

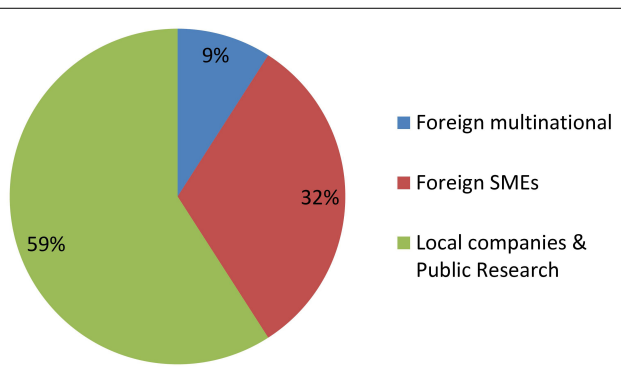

FIGURE 3 | NBT (non-GMO) products by developer profiles. See text for details.

5 year has it been feasible that occasionally a local company or a foreign SME is able to deregulate a GM crop.

In contrast, **Figure 3** shows that research institutes and/or local SMEs are responsible for about half of NBT products presented to the regulatory authorities, from the very beginning. In these cases, the whole process of product development, deregulation and commercialization is in the hands of such local actors from Argentina, a developing country. Regarding the other half of the cases, most of them correspond to products developed by foreign SMEs, and finally a small proportion was presented by MNCs.

Number of Developers

Figures 4, **5** report the number of different developers (companies or institutions) corresponding to each one of the developer profiles as described previously. MNCs have been subdivided into those commercializing veterinary vaccines or those dealing with GM crops. In regards to the latter, reckoning was based on currently existing business entities, thus taking into account the recurring processes of merging and acquisitions that took place during the last three decades in the field of GM crops.

Approved GMOs developed by MNCs are numerous, but concentrated in only four companies (**Figure 4**). In contrast, a few GMOs were deregulated by the public sector and SMEs and almost each one is owned by a different company.

Figure 5 shows that the number of different applicants for organisms improved using NBTs is already higher than the number of applicants that deregulated GMOs. This must be considered in perspective with the fact that NBT cases represent only a 3-year period of time, against a 23-year period for several dozens of GMOs.

In terms of product concentration, NBTs are typically distributed at 1–2 products per applicant, with only one outlier being an important Argentinean public research institute that holds 23% of applications. In contrast, the distribution of authorized GMOs per applicant is very uneven, with a handful of MNCs concentrating most products, including a single one that deregulated 40% of all GM crops.

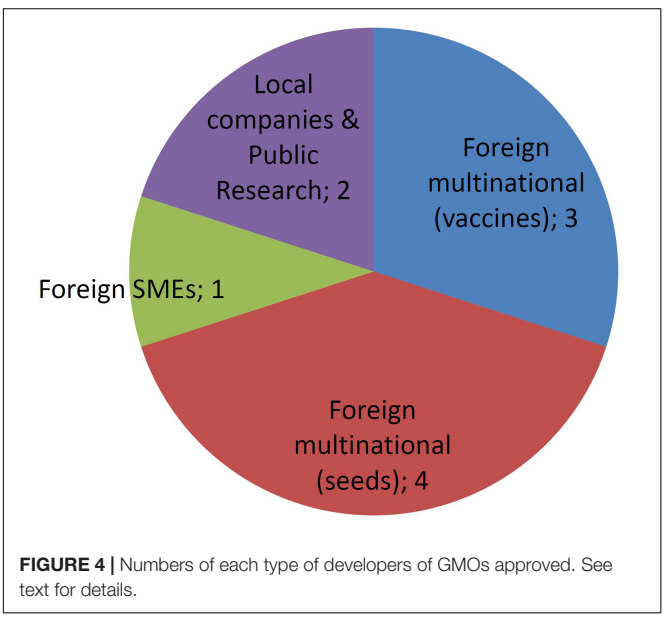

FIGURE 4 | Numbers of each type of developers of GMOs approved. See text for details.

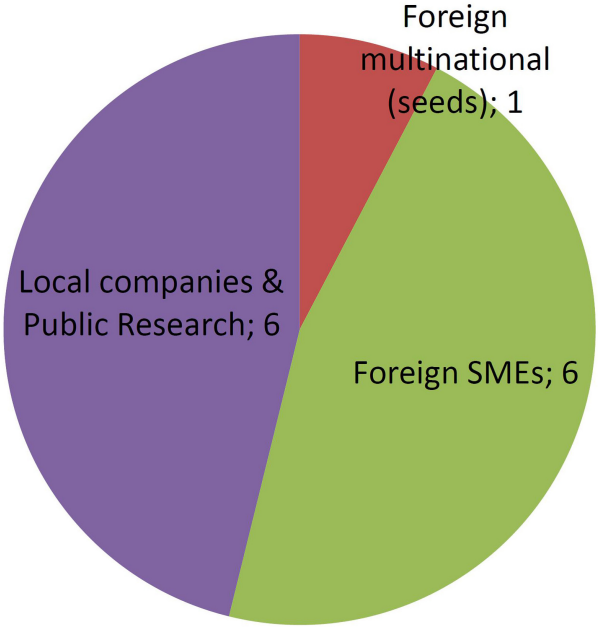

FIGURE 5 | Numbers of each type of developers of NBT (non-GMO) products. See text for details.

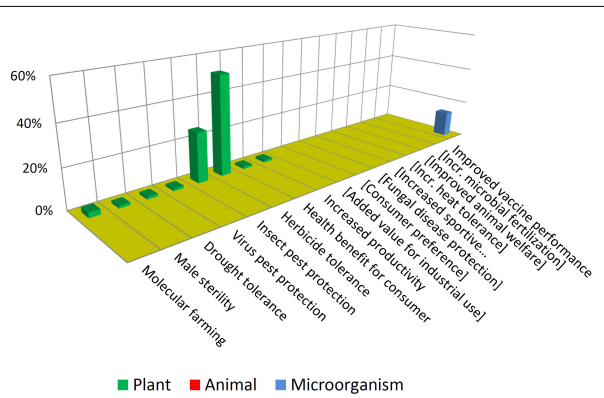

FIGURE 6 | Introduced traits in GMO products. See text for details.

From this insight, the market of crops and other agricultural organisms improved by NBTs is anticipated to be less concentrated in terms of proprietor entities. Therefore, it should be more competitive and more diversified, both in terms of commercialization conditions (cost, license conditions, etc.) as well as in regards to the availably of technical options in terms of traits and crops (the latter is explored next).

Traits

Figure 6 illustrates that most GMOs that have reached commercialization are plants having traits of herbicide tolerance

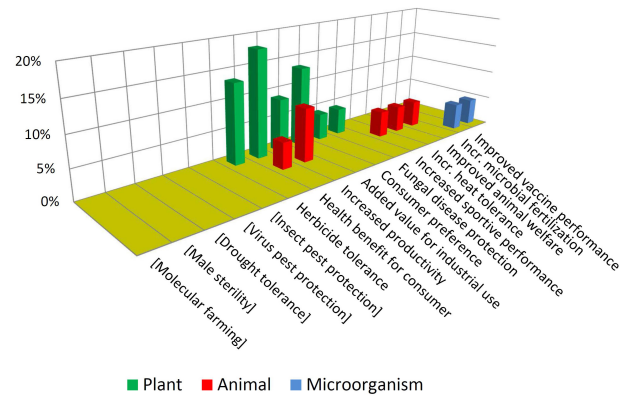

FIGURE 7 | Introduced traits in NBT (non-GMO) products. See text for details.

and insect protection. Further to this, such traits are present mostly in three crops: maize, soybean and cotton. This situation is common to almost all countries growing GM crops (ISAAA, 2019).

Such products that consist in crops that are ubiquitously cultivated in large acreages combined with not-novel, unspecialized traits are sometimes referred to as "blockbusters" (Gewin, 2003; Stokstad, 2004). This expression captures the concept that MNCs tend to focus on conservative strategies involving crops and traits whose seeds may be demanded by farmers in high quantities and in many locations of the world. There are only a few "non-blockbusters" among approved GMOs. This includes drought tolerance, virus protection and even a case of "molecular farming" (Spiegel et al., 2018), consisting in a cheese-making enzyme produced in plants.

In contrast with the above, **Figure 7** shows that NBT products display a higher diversity in terms of traits and biological kingdoms. Such a difference may become bigger in the future, considering that the GMO cases are the result of a pipeline that has been stabilized over many years, while the unfolding of the NBT pipeline has begun much more recently.

Note that some traits which are not present among approved GMOs but are present among the NBTs have been included (enclosed in brackets) in **Figure 6**, and *vice-versa* in **Figure 7**, for a better comparison between the two figures.

It is also interesting to compare which traits are common or not to both groups. For instance, herbicide tolerance is significantly present for both technological options. This may be driven by its high demand as a blockbuster trait. In addition, for many crops there are no herbicide tolerant varieties, because of a lack of success regarding spontaneous mutations and "sociotechnical resistance" (Thomas et al., 2017) to GMOs; in such cases a gene-edited tolerant mutant may appear as promising alternative, worthwhile to be developed (Zhang et al., 2019).

In contrast, pest protection traits against insects and viruses, which are currently achieved by Bt proteins and RNA interference in GMOs, are not represented among NBT products; however, NBTs do include one case of protection against a fungus. This is a trait that has been repeatedly achieved by transgenesis but no GMO is commercially available yet; it has been suggested that the uncertainties and complexities of deregulating a fungus-protected GM crop have delayed such innovation (Cornelissen and Melchers, 1993; Wally and Punja, 2010). Perhaps in the case of NBTs a more affordable regulation would allow to reach the total investment required for delivering such kinds of traits to the market.

Drought tolerance is an intense field of development for both GMOs and NBTs (Cominelli and Tonelli, 2010; Jaganathan et al., 2018; Rodrigues et al., 2019), likely fostered by the increasing challenges derived from climate change. Although drought tolerance is currently represented only among GMOs, likely this will be also a target using NBTs, which nevertheless already includes one case pertaining to a different abiotic stress: heat tolerance.

Lastly in the case of molecular farming, such as industrial enzymes or pharmaceuticals produced in plants or animals, since this may only be possible by inserting genes from other organisms, such cases will always be considered GMOs.

Distribution by Organism Type

By comparing **Figures 8**, **9** it can be seen that diversity of organisms is already greater in NBT products than GMOs, grouped in terms of agricultural categories. This is because of differences in regards to (a) the presence of animals among the NBT cases, being absent among deregulated GMOs, (b) microbial products, where live and viable vaccines are present in both, but NBT products in addition include microbial agricultural "bioinputs" (Kour et al., 2017), and (c) diverse categories within the plant kingdom. Categories that are not represented in a figure but still shown for comparison with the other are enclosed in brackets.

Not surprisingly, GM crops are dominated by oilseeds, cereals and fiber crops, which in fact are represented by only one species each: soy, maize, and cotton. In contrast, albeit with lesser cases the NBT products are more dispersed among a higher number of crop categories and species. Interestingly, no fiber crops improved using NBTs have been presented yet. This

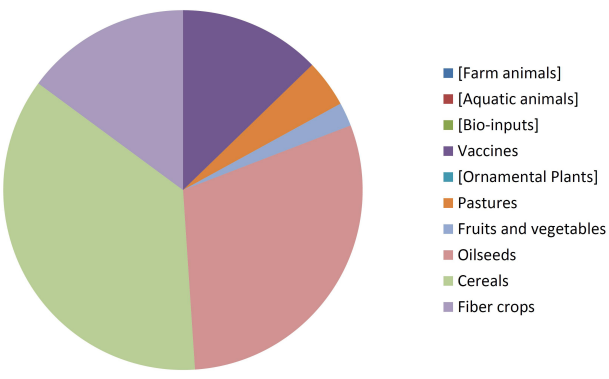

FIGURE 8 | Approved GMOs distributed by type of organism. See text for details.

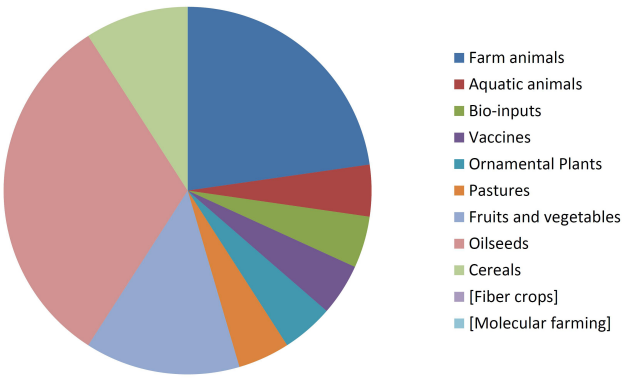

FIGURE 9 | NBT (non-GMO) products distributed by type of organism. See text for details.

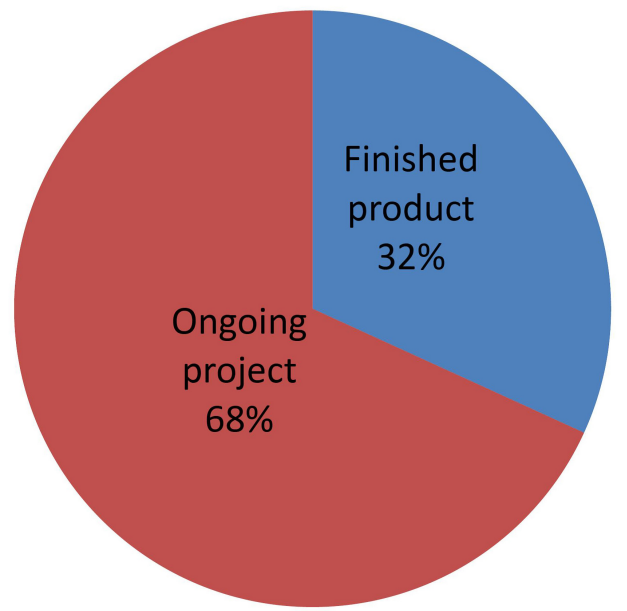

FIGURE 10 | Distribution of NBT (non-GMO) products, classified by state of development. See text for details.

might be expected, though, since cotton is a less-problematic kind of GMO in terms of trade issues and public perception, as it is a cash crop mainly used for obtaining non-edible textile material. Therefore, there might be less incentive for finding alternative innovative breeding technologies for cotton compared with other species.

State of Development

Figure 10 shows a distribution of NBT products that have been submitted to the Argentine regulatory system, classified according to their level of development. "Finished product" means those whose breeding process is complete and the product has been fully studied at the phenotypic and molecular levels. Such products are in a position to receive a final determination of "non-GMO" status.

Conversely, "Ongoing projects" are those where the final characterization of the product is not fully available. As described by Whelan and Lema (2015), developers at this stage are able to request a formal preliminary analysis based on the expected characteristics of the final product, which shall be re-confirmed later when a full phenotypical and molecular characterization becomes available.

Many developers are requesting this option of preliminary analysis. This is presumably because they find it very valuable for planning and taking decisions on continuing with the project, as well as for attracting funding once they can estimate the regulatory costs with more reliability. The option of receiving a formal preliminary analysis is likely playing an important role in fostering investment and development of NBT products.

Usage of Gene-Editing Within NBTs

Gene editing, especially using CRISPR-Cas nucleases, is attracting a lot of interest for breeding and other purposes (Jaganathan et al., 2018; Chen et al., 2019). Argentine regulation for NBTs of course includes products obtained by genome editing and, not surprisingly, it is the most commonly applied NBT of the cases submitted to the regulatory system in Argentina. See **Figure 11**.

For this analysis, we have considered gene editing to include techniques encompassed by the terms "site-directed-nuclease" (SDN) of types 1, 2, and 3, as well as "oligonucleotide-directed-mutagenesis" (ODM), according to the definitions by Lusser et al. (2011). Counter-examples of NBTs that are not gene editing techniques include epigenetic modification

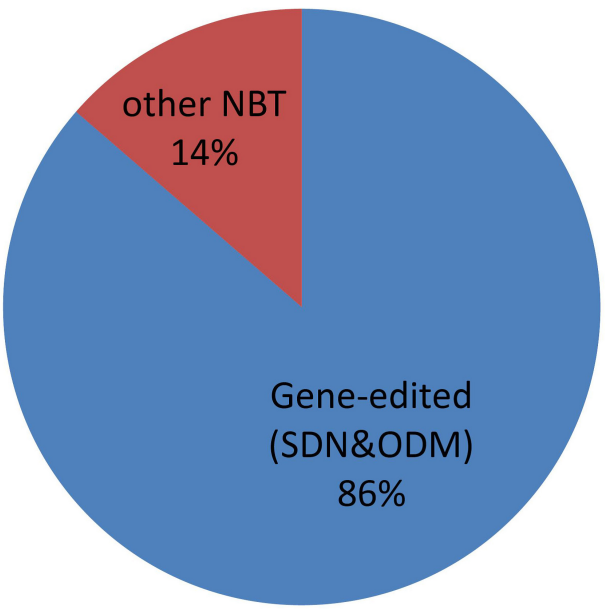

FIGURE 11 | Usage of Gene-editing within NBTs. See text for details.

(Álvarez-Venegas and De-la-Peña, 2016), reverse breeding (Dirks et al., 2009), etc.

Although genome editing represents a vast majority, there is also a proportion of other NBT products. It is important to realize that this is a rapidly evolving field, where regulation must be designed to withstand the test of time (i.e., technical advances) as much as possible. As a demonstration of this, it can be pointed out that the term "NBT" was coined -for regulatory purposes- 1 year *before* the first CRISPR-Cas tool became known, but nowadays it has become the dominant technology within NBTs. Novel gene editing techniques are published and patented every month, and their similarities and/or differences with other NBTs are more difficult to define, for instance with CRISPR-Cas tools adapted to perform epigenetic interventions (Pickar-Oliver and Gersbach, 2019).

In this sense, it is important to highlight that the Argentine regulation has been scripted without the need of inserting a list of specific techniques. Consequently, it is not restricted to the particular technological configurations available at the time the regulation was drafted. Therefore, it avoids delaying or discouraging incremental innovations as they appear later on.

CONCLUSION

This article has compared apparent trends amongst technologies presented to the Argentine regulatory system for agricultural biotechnology. This was done with the purpose of detecting emerging opportunities for strengthening local innovation processes in the agricultural sector. This is just an initial study, because further STS are needed for a more broad and comprehensive research agenda on innovations enabled by gene editing and other NBTs. Such an agenda should include (a) comparative case studies of specific products having the same trait but obtained through different breeding technologies (such as Bullock et al., 2019), as well as (b) quantitative estimations of the macroeconomic impacts derived from NBT products altogether.

According to the preliminary evidence presented here, the regulatory approach adopted in Argentina is already stimulating local innovation processes. Noticeable changes include an increase of technology developers/providers and the diversification of products; the potential impacts appear to be higher for breeding niches that have not been explored yet by (commercial) agricultural biotechnology.

It has been postulated already that genome editing will be a democratizing technique; however, these assertions were based on qualitative reasoning or very early milestones of technology development (Jackson et al., 2019). In this work we present evidence for this trend that is collected closer to the actual use of this technology. A corollary is that genome editing should be less prone to the criticism/protectionism raised against GM crops from allegations that they could affect "food democracy" (Friedrich et al., 2019) or food security/sovereignty.

Moreover, it can be proposed that a reasonable regulation for gene editing, in particular, will have an immediate and direct effect on the agricultural innovation system, particularly if it allows improving the predictability of regulatory costs for innovative products. Besides this, the investment of time and money required in order to meet regulatory requirements may be more attainable compared with the option of developing the same traits using GMO technology.

Gene editing is perhaps the newest paradigm shift of the present-day industrial revolution that encompasses biotechnology (Rifkin, 1998; Karan, 2016). The emergence of a technological paradigm creates a context for establishing new development policies that expand opportunities for local actors (Freeman and Pérez, 2003). Taking into account that opportunities for economic development are a mobile target, sometimes linked to paradigm shifts (Pérez, 2004), and genome-edited products constitute a window of opportunity for developing countries. This opportunity is also available to developed countries where the first wave of local development based on GMOs crashed against a barrier of over-regulation (Jorasch, 2019). Not surprisingly, the forerunner Argentine regulation has inspired another eight countries in Latin America to enact similar regulations in less than 4 year, and is quite in line with regulatory developments occurring recently in countries from Africa, Asia and Oceania.

A more dynamic market of innovation creates opportunities to expand the supply of local technologies. This can strengthen the agricultural innovation system, because it allows new actors to enter through the window of opportunity. The technological shift makes it easier for SMEs and public R&D laboratories to develop new products on their own, thus expanding the market, both in terms of participants and products. In addition, the reduction in the scale of production necessary to reach profits can favor the development of local economies.

In conclusion, the results of this prospective study suggests that gene editing could drive further innovation and "democratization" of agricultural biotechnology, thus leading to increased productivity and economic development, if managed under effective regulatory processes.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request 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.

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BRIEF RESEARCH REPORT
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Biosafety Measures, Socio-Economic Impacts and Challenges of Bt-brinjal Cultivation in Bangladesh

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This study surveyed the onsite biosafety measures adopted by the farmers cultivating Bt-brinjal, the socio-economic impact, and the challenges of Bt-brinjal cultivation in Bangladesh through interviews of 101 farmers from 26 Upazila (administrative region) under 20 Districts. Bt-brinjal 2, released by Bangladesh Agricultural Research Institute (BARI), is cultivated by 35% of the surveyed farmers. It was revealed that 52% of farmers maintained border crops. Among the growers, 52% informed that they disclose to the buyers that they are selling Bt-brinjal while selling in the open market where no product is traditionally labeled. Most of the farmers (71%) use Bt-brinjal plant debris as animal feed. Farmers (60%) received training on biosafety of Bt-brinjal cultivation. According to 85% of farmers, Bt-brinjal cultivation improved insect control. The farmers (77%) agreed that Bt-brinjal reduced labor and chemical costs and 75% of the farmers found increased yield and 72% of them found enhanced income by Bt-brinjal cultivation. However, 25% farmers informed that they did not get increased yield due to incidence of secondary insects. Most of the farmers (89%) perceive that cultivation of Bt-brinjal improved quality of brinjal. Furthermore, 59% of the farmers opined that price was reduced due to Bt-brinjal cultivation. The farmers also believe that Bt-brinjal cultivation reduced pesticide use (97%) and concern of insecticide use (96%) and hence they consider Bt-brinjal safer for human health (96%). However, to harvest the benefits of modern biotechnology, proper management of the biosafety in Bt-brinjal cultivation and labeling of Bt-brinjal during marketing should be maintained properly.

Keywords: biosafety, Bt-crop, eggplant, farmers' perception, fruit and shoot borer, pest management

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INTRODUCTION

Brinjal and Fruit and Shoot Borer (FSB)

Brinjal (*Solanum melongena L.*), also known as eggplant, is a popular multiuse vegetable cultivated in Asian countries, including Bangladesh. In Bangladesh, it is grown by about 150,000 farmers in 50,000 hectares of land, throughout the year in both the winter and summer seasons. The eggplant fruit and shoot borer (FSB) is responsible for the chronic and widespread infestation and considered the biggest constraint to eggplant production throughout Asia. FSB has become a major

and regular pest of brinjal causing damage to even 30–50% of fruits or more in India, Bangladesh, Malaysia, Thailand, Burma, Sri Lanka, Laos, South Africa, Peoples Republic of the Congo. In severe cases, the infestation levels may exceed 90% and causing yield loss of up to 86% in Bangladesh (Ali et al., 1980). It affects the quality and quantity of fruits (Mall et al., 1992) rendering the fruits difficult to sell on the market and contains significantly less vitamin C (Abrol and Singh, 2003; Ghosh et al., 2003).

Fruit and Shoot Borer (FSB) Control Measures

Farmers use tons of chemical pesticides annually to control pests that cause economic damage to crops. It was reported that 98% of the farmers rely solely on insecticide applications (Karim, 2004). The farmers spray insecticide almost every alternate day with as many as 84 applications in a cropping season (BARI, 1994). Not only in Bangladesh but also the Philippines, damage by FSB resulted in 80% yield loss of fruits and the control relies primarily on frequent applications of insecticides (Francisco, 2009). Consumers wish to avoid eating food that has been treated with pesticides because they are afraid of potential health hazards. The discharge of agricultural wastes from excessive use of pesticides and fertilizers can poison the water supply and cause harm to the environment. Moreover, pesticides are often applied without the appropriate protective equipment, resulting in high and prolonged exposures to farmers. Consequently, farmers suffer numerous health problems resulting from direct exposure to pesticide during handling and spraying (Rahman, 2000; Wilson and Tisdell, 2001). In Bangladesh, almost all farmers experienced sickness related to pesticide application, and 3% were hospitalized due to complications related to pesticide use (Alam et al., 2003). In India, 43% of the brinjal farmers suffered from health hazards due to various complexities related to pesticide application (Kolady and Lesser, 2005). Growing genetically modified (GM) Bt-crops (transgenic crops that produce the same toxin as the bacterium *Bacillus thuringiensis* in the plant cell, thereby, protecting the crops from pests) can reduce the application of chemical pesticides and the cost of bringing a crop to market (Moellenbeck et al., 2001).

Release of Bt-brinjal and Cultivation

On 30 October 2013, Bangladesh approved the official release of four genetically modified, varieties of insect-resistant Bt-brinjal for seed production and initial commercialization. Bt-brinjal cultivation began in early 2014 in the spring season. The seedlings of four Bt-brinjal varieties were distributed to 20 small brinjal farmers on 22 January 2014. The farmers planted Bt-brinjal in a total area of 2.6 hectares in four representative regions of Gazipur, Jamalpur, Pabna, and Rangpur where these varieties are well-adapted and carefully monitored. Bt-brinjal-1 variety, popularly known as Uttara, was planted in Rajshahi region; Bt-brinjal-2 (Kajla) in Barisal region; Bt-brinjal-3 (Nayantara) in Rangpur and Dhaka regions; and Bt-brinjal-4 variety, Iswardi/ISD006, was planted in Pabna and Chittagong regions of the country. The Bangladesh Agricultural Development Corporation (BADC) in collaboration with BARI distributed seeds to farmers in the Kharif (Summer) season 2014. The government of Bangladesh planned to bring 20,000 hectares (40% of total 50,000 hectares) of land across 20 districts under Bt-brinjal cultivation. There are an estimated 150,000 brinjal farmers in Bangladesh, out of which 27,012 (∼17%) farmers are enjoying the benefits of the technology in 2018 (Shelton et al., 2018). Bt-brinjal is the first genetically modified (GM) Bt-food crop to be commercially cultivated in Bangladesh and in the world. Hence, the success of the Bt-brinjal cultivation, farmers' profitability, the safety of environment and health and handling the future challenges efficiently can affect development and release of future genetically modified crops in Bangladesh, and other countries where biotechnology can play a vital role for food security and environmental safety.

Biosafety in Bt-brinjal Cultivation

Handling transgenic crops in various stages require biosafety measures to ensure the conservation and sustainable use of biodiversity. Department of Environment in Bangladesh is responsible for ensuring biosafety measures through the implementation of Biosafety Rules and Guidelines (Biosafety Guidelines of Bangladesh, 2005; Anonymous, 2006b). The department helped the government to make decisions on genetically modified organisms (GMO) to be used in various conditions from lab to placement into the market. Although the government endorsed various uses of GMOs, there is no comprehensive information how the biosafety rules and guidelines are applied or followed by the farmers and benefits they are getting and also the challenges to be faced. Therefore, an initiative was taken to study the present status of the biosafety measures in post-release cultivation of Bt-brinjal to meet this information gap.

METHODOLOGY

The study was conducted at 26 Upazila under 20 districts (**Table 1** and **Figure 1**). The areas were randomly selected and were representative of all parts of Bangladesh. This study was conducted between March to May 2018 and consisted of interviews with 101 Bt-brinjal farmers. The surveyors visited Bt-brinjal cultivated field, talked to the farmers and consumers and collected data. An inclusion criterion was set for those farmers directly cultivating Bt-brinjal and focal farmer (farmers having regular contact with extension support staff), under the Department of Agriculture Extension (DAE). Farmers who had no land under DAE supervision were excluded from this survey.

We designed a questionnaire based on published literature (Anonymous, 2006a; Talukder, 2012) and the authors' experiences in the field of biosafety. The questionnaire originally designed in English was translated into Bangla, the national language for the easy understanding of the farmers. Data were collected through a survey by face-to-face interviews with farmers and field observations during farming activities. The farmers were informed about the purpose of the study, and verbal consent was taken before the interview. The questionnaire

TABLE 1 | Name of the selected location to survey of Bt-brinjal cultivating area.

Sl. No.	Name of district	Name of Upazila
1	Bogura	Gabtoli
2	Sylhet	Sylhet Sadar
		Gohainhat
		Bianibazar
3	Dinajpur	Dinajpur Sadar
4	Kushtia	Kushtia Sadar
5	Bager hat	Mollarhat
6	Moulovibazar	Juri
		Borolekha
7	Pabna	Pabna Sadar
8	Barishal	Babuganj
9	Khulna	Kotiaghata
10	Jessore	Jhikorgacha
11	Potuakhali	Dumki
12	Madaripur	Madaripur Sadar
13	Chittagong	Chittagong Sadar
14	Mymensingh	Gouripur
		Trishal
		Muktagacha
15	Tagurgao	Kaliadangi
16	Rangpur	Mithapukur
		Gongacora
17	Vola	Chorfashion
18	Rajshai	Puthia
19	Gaibandha	Polashbari
20	Chadpur	Kachua

consisted of four pages with 38 items. All items were rephrased as statements or a dichotomous statement (yes-no).

RESULTS

Respondents' Age, Educational and Socio-Economic Attributes

The study was conducted on 101 farmers who cultivate Bt-brinjal in 20 districts of Bangladesh. The farmers represented the mid-age group of 31–50 years followed by above 50, and below 30, respectively with diverse educational levels in the order of SSC (Secondary School Certificate) > write their name only > illiterate > above HSC (Higher Secondary Certificate). This study found that 67% of the Bt-brinjal farmers are subsistence farmers and they have less than 0.1 ha of land for Bt-brinjal cultivation. Only 5% of the Bt-brinjal farmers grew Bt-brinjal in 0.5–1.0 ha of land (**Table 2**). It shows that all the growers are marginal farmers having little or no profit from the farming but enjoying only a minimal livelihood. A recent report also found that nearly half of all brinjal farmers in both treatment and control groups are small farmers operating 0.5 to 1.49 acres of land (Ahmed et al., 2019). According to them, the second largest group is the medium farmer category, working 1.5 to 2.49 acres. The annual income of the farmers growing Bt-brinjal varied considerably. Most of the farmers belonged to the low-income group having their income below 20,000 Tk (Bangladeshi Taka) per annum. It reveals from **Table 2** that 36% had an annual income below 10,000 Tk and 32% of the farmers had a yearly income below 20,000 Tk but above 10,000 Tk. However, 20% of the farmers had a higher income above 30,000 Tk per year.

Farmers' Training for Bt-brinjal Growing

The study surveyed whether the farmers received any training that covered the biosafety measures to be taken for growing genetically modified Bt-brinjal and the process of cultivation of Bt-brinjal. Majority of the farmers (60%) under this study had exposure to training, while 40% had no training (**Table 2**). The farmers informed that BARI arranged 1 day training on Bt-brinjal cultivation for a limited number of farmers. It was reported that BARI, DAE and International Food Policy Research Institute (IFPRI) organized training of trainers, officers and farmers during 2017 covering various aspects of Bt-brinjal cultivation (Ahmed et al., 2019). Before the first release of Bt-brinjal in 2014, farmer training was conducted by BARI. More recently, the Department of Agricultural Extension (DAE) and the Agriculture Information Service (AIS) have become involved in training and distributing information on Bt-brinjal. However, the training was free of cost and did not cover all the farmers. The farmers without training hope that they should be given a minimum training on the safety and management of Bt-brinjal.

Management Practices of Bt-brinjal Cultivation

Popularity of the Variety

Farmers of Bangladesh are cultivating four varieties of Bt-brinjal. Among the varieties, Bt-2 (Kazla) variety is grown by 35% of the targeted farmers, while 29% targeted farmers cultivate BARI Bt-brinjal 4 (ISD006). Only 7% of farmers are growing mixed; more than one varieties (**Table 3**). The Government of Bangladesh distributed seeds of four Bt-brinjal cultivars to different regions based on the history of consumers' and farmers' choice of the traditional counterpart of that GM brinjal cultivated. However, the farmers' preference depends on the region of the country and the consumers' choice over many years.

Management of Border Crops

The management of border crops is one of the most important safety aspects of GM crop cultivation. Cultivation of non-GM crop as a border crop around Bt-crop is advised for insect resistance management. In the case of Bt-brinjal, cultivation of 5% non-Bt-brinjal is necessary. It was found that more than half of the respondent farmers (52%) maintain border crop while growing Bt-brinjal in their fields. However, 45% of the farmers do not grow border crop, and only an insignificant percentage of farmers (<3% farmers) are unaware about the importance of growing border crops around the Bt-brinjal fields (**Table 3**). Although, more than 50% of farmers manage border crop, nearly equal percentage of farmers either do not manage or unaware of the matter. The farmers grow mostly non-Bt-brinjal as border crops. In 41% of the cases, farmers use non-Bt-brinjal variety ISD 006 while 24% of the farmers use local

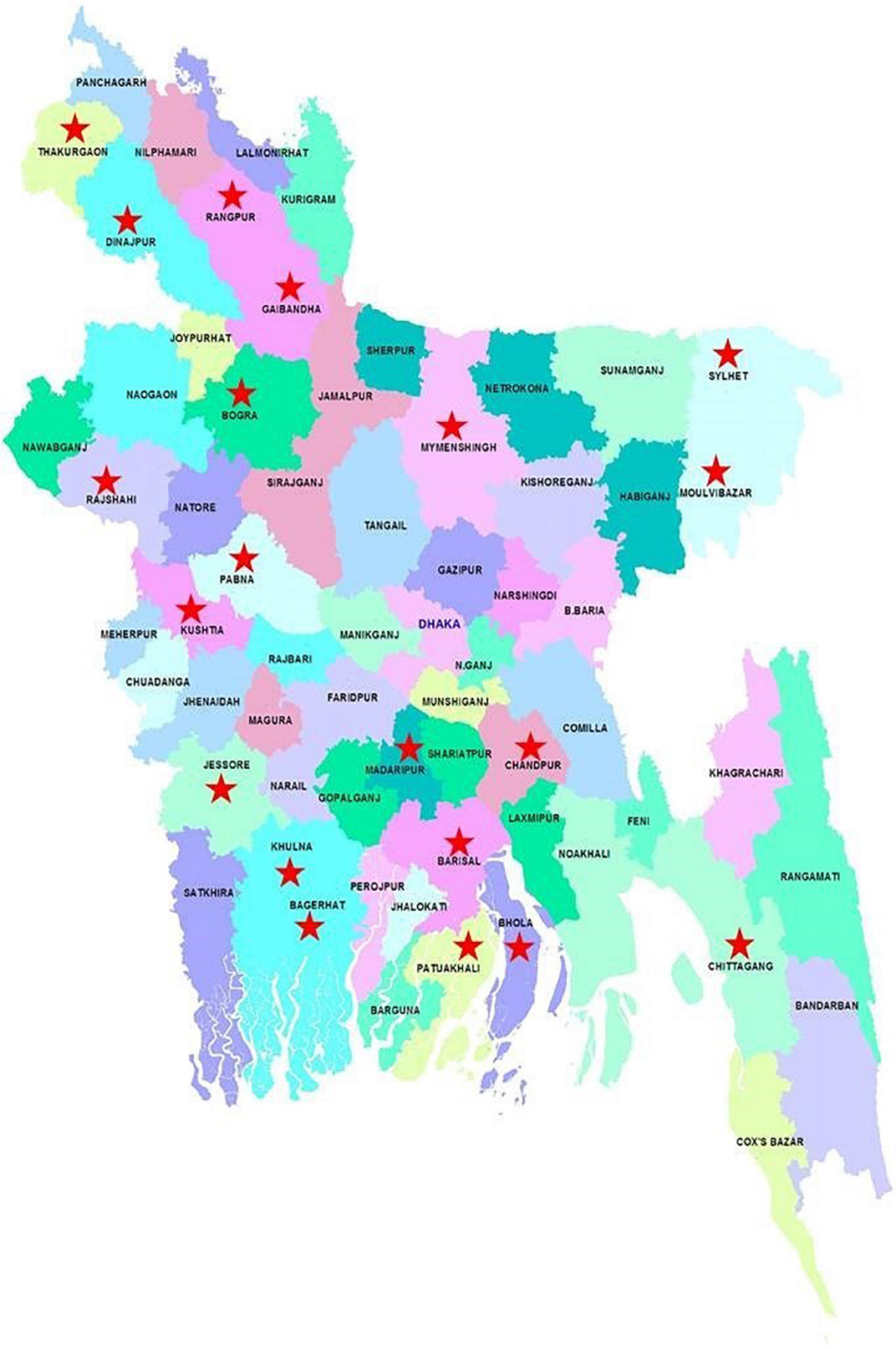

FIGURE 1 | A map of Bangladesh showing the locations (*) where the survey was conducted. The map is a modification from that available in http://mapsof.net/uploads/thumbnails/500/bangladesh.png.

TABLE 2 | Basic information of the Bt-brinjal farmers.

Factor	Category	Percentage (%)
Age (years)	up to 30 years	15
	31–50	60
	> 50	25
Educational level	Illiterate	11
	Sign only	20
	SSC (Secondary School Certificate)	60
	HSC (Higher Secondary Certificate)	10
Farm size (ha)	Up to 0.1	67
	0.1 to 0.5	28
	0.5 to 1.0	5
Annul income	1,000–10,000 (low)	36
Bangladesh Taka (Tk)	11,000–20,000 (low)	32
	21,000–30,000 (medium)	12
	31,000–40,000 (high)	6
	> 41,000 (high)	14
Training on	Yes	60
Bt-brinjal cultivation	No	40

TABLE 3 | Management practices of Bt-brinjal cultivation.

Factor	Category	Percentage (%)
Popularity of the varieties	Bt-brinjal −1	11
	Bt-brinjal −2	35
	Bt-brinjal −3	11
	Bt-brinjal −4	29
	Combine	7
Border crop management	Yes	52
	No	45
	No knowledge	3
Crop security management	Fencing	50
	Watchman	20
	No need	13
Pest management	Yes	58
	No	42
Harvesting the Bt-brinjal	Mix up Non-Bt-brinjal	62
	Non-mix up Non-Bt-brinjal	38
Labeling the Bt-brinjal for sale	Yes	52
	No	48
Debris management	Animal feed	71
	Burning	9
	Others	21

brinjal as border crop (**Figure 2**). It was found that 50% of the farmers used to fence around the Bt-brinjal field to protect the crop from animals. The others kept watching men for the protection of the crops (**Table 3**). BARI is continuing its effort in training focusing on the unique aspects of Bt-brinjal, mainly the requirements to plant a refuge of non-Bt-brinjal and the need to manage other "sucking insects" (Shelton et al., 2018). The survey indicated that 58% of farmers practised pest resistance management and applied insecticide to control insects other than FSB, while 42% did not apply any insecticide (**Table 3**). The data reveals that despite cultivating shoot and fruit borer

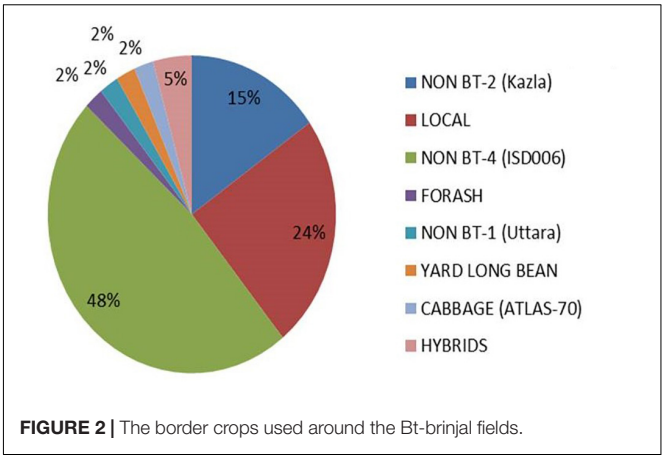

FIGURE 2 | The border crops used around the Bt-brinjal fields.

resistant variety, 58% of the farmers are still afraid of other minor insects like mites and aphids. They want to keep their crop from minor insects. Therefore, they are using insecticides to protect brinjal from any loss.

Labeling of Bt-brinjal

The study results indicated that the majority (62%) of the farmers mix the Bt-brinjal with non-Bt-brinjals during harvesting (**Table 3**). In that case, the labeling cannot be done properly. Only 38% of the farmers are careful about separating the Bt-brinjal from non-Bt-brinjal. It indicates that the farmers are not very careful about the harvesting practice of the Bt-brinjal that is necessary for the labeling of Bt-brinjal.

In Bangladesh, labeling is rarely done for vegetables in the open market. It was found that 52% of Bt-brinjal growers inform the buyers that the brinjal is genetically modified while taking the product for sale in the market. In fact, they sold brinjal in open market where no labeling is traditionally practised in Bangladesh. The farmers informed that they just disclosed the buyers that the brinjal they are selling is Bt-brinjal. On the contrary, 48% growers did not mention the buyer that they are selling Bt-brinjal probably due to lack of training and knowledge or failure of understanding about the importance of the matter (**Table 3**).

Plant Debris Management

Management of the debris of the plants after harvest of Bt-brinjal is an important biosafety issue during containment and contained trials of GM crops. However, it is important to manage the debris of the plants after harvest of Bt-brinjal for biodiversity reason. Most of the farmers (71%) used the plant debris as animal feed, while 9% of farmers follow incineration or burning of the debris (**Table 3**). The rest 20% use them in various purposes like using as fuel in the kitchen or leave the debris in the field. The farmers are supplied with fresh seeds, and they normally do not keep the seeds for next year.

Benefits of Bt-brinjal Cultivation by Farmers

Farmers benefit is the main component of GM brinjal cultivation as farmers are the primary stakeholder. Farmers will not cultivate Bt-brinjal if it is not profitable for them. In this survey, 85% of the farmers informed that cultivation of Bt-brinjal improved

insect control (**Figure 3**). In comparison, 15% of the farmers disagreed with this opinion because there were other minor insects (e.g., whitefly) that caused damage to the Bt-brinjal, and Bt-brinjal had a higher number of leaves that caused hindrance to control insects. The basis of their disagreement was the presence of some minor insects in the field. Nearly 100% control of FSB by Bt-brinjal in the Philippines was reported (Hautea et al., 2016) with no negative impacts on non-target arthropods (Navasero et al., 2016). While in Bangladesh, as many as 6,500 farmers grew Bt-eggplant in 2017 and reaped its benefits (Shelton et al., 2017). Similar results were published from agronomic and socioeconomic studies conducted in Bangladesh (Shelton et al., 2018). Cultivation of Bt-brinjal reduced labor and chemical costs. Prodhan et al. (2018) compared the impacts of four Bt-brinjal varieties and conventional brinjal. They found a 0–2% fruit infestation of FSB among the Bt-brinjal varieties versus a 36–45% infestation in conventional brinjal varieties. Ahmed et al. (2019) reported FSB infested only 2% of all Bt-brinjal plants grown by the treatment farmers; by contrast, 34% of all ISD-006 brinjal plants grown by the control farmers were infested by FSB. These reports further demanded that Bt-brinjal has been successful in repelling FSB infestation and had no impact on non-target beneficial insects. A previous study by Rashid et al. (2018) assessed the impacts of four varieties of Bt-brinjal during the 2016/17 winter season and found that net returns were Tk 179,602 per ha for Bt-brinjal versus Tk 29,841 per ha for conventional brinjal (six times larger for Bt-brinjal farmers). Majority of the farmers (77%) agree with this opinion. In comparison, 23% told that there was no reduction of labor and chemical costs because they had to spray several times to control minor insects (**Figure 3**). These farmers reported that there was an incidence of secondary insects, and they had to spray insecticides to control those pests; thus, there was no considerable reduction in labor and chemical costs. Ahmed et al. (2019) claimed that overall, the cost of Bt-brinjal production per ha dropped by about 11% and cost per kg reduced by 31%. Again, 75% of the growers found increased yield due to growing Bt-brinjal while 25% of farmers informed that there was no increase in yield due to the incidence of secondary insects. A recent study reports that net yields were ∼40% higher for Bt-brinjal farmers compared to the conventional brinjal (Ahmed et al., 2019). The farmers were asked if they had an increase in income by Bt-brinjal cultivation; 72% growers replied that they were benefitted with an increase in income by Bt-brinjal cultivation. However, 28% of the growers replied that they did not find any increase in income because they did not get a good price of the brinjal (**Figure 3**). According to a recent report, the cost of production drops, mainly driven down by reduced pesticide costs, and revenues increase, mainly because of higher yields of Bt-brinjal and higher price. Increased production and a 10% reduction in costs, lead to a substantial increase in profits from cultivating Bt-brinjal that also conveys significant health benefits, both human and ecological while raising farmer incomes (Ahmed et al., 2019).

Farmers' Perception of Bt-brinjal

Farmer satisfaction is a very important issue of Bt-brinjal cultivation as they are the most important stakeholder. If the farmers are not satisfied with Bt-brinjal, the cultivation is meaningless. This survey found that 89% of the farmers believe the cultivation of Bt-brinjal improved quality of brinjal, while 11% of farmers disagreed with this opinion (**Figure 4**). According to them, the taste was not the same as the non-Bt-brinjal. This disagreement might be the reflection of the differential performance of four varieties in various locations and individual choice of the farmers. Again, 59% of farmers opined that price was reduced due to Bt-brinjal cultivation because of higher production. In comparison, 41% did not find a reduction in price, so they believe that the adoption of Bt-brinjal cultivation did not reduce the price (**Figure 4**). The farmers (97%) informed that Bt-brinjal cultivation reduced pesticide use that they knew from the farmers and vegetable sellers and alleviated the concern of insecticide use (96%). Hence, they consider Bt-brinjal safe for human health (96%). However, 2–4% of the farmers did not agree with the above opinions.

A recent study found that Bt-brinjal released by Bangladesh government has cut toxicity of pesticides used by 41% and the farmers increased revenues by 27%. BARI scientists conducted a study in 35 districts during the 2016–2017 cropping season and reported that the farmers saved 61% of the pesticide cost

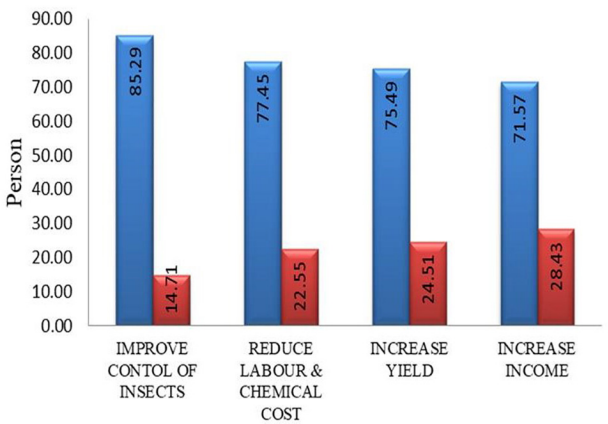

FIGURE 3 | Benefits of Bt-brinjal cultivation by farmers.

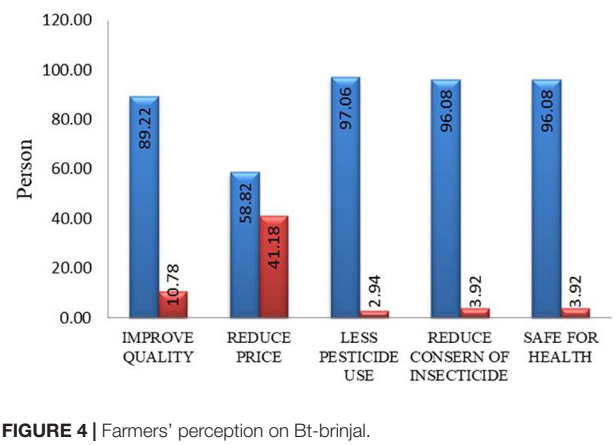

FIGURE 4 | Farmers' perception on Bt-brinjal.

compared to non-Bt-brinjal farmers and received higher net returns (unpublished, cited from Ahmed et al., 2019). Previous studies have shown that Bt-brinjal gave control of FSB and reduced insecticide use, with ultimate economic, health, and environmental benefits (Shelton et al., 2018). Because, it provides improved food safety, a more consistent supply of a highly nutritious vegetable, and less insecticide in the environment (Shelton et al., 2017). This study unveiled the fact that the farmers are happy with Bt-brinjal cultivation (**Figure 5**).

CHALLENGES AHEAD

Every new approach faces challenges. Commercial cultivation of GM Bt-brinjal past few years has also generated concerns about its potential impacts on the environment, biodiversity and human and animal health. Ecological risk assessment of transgenic crops, issue of gene flow, development of secondary pest resistance and environmental risks involved with pollen flow are some of the issues related to any GM crop commercialization (Craig et al., 2008). People are confused about the risk that Bt-brinjal may pose to human health and the environment; the adequate follow up of guidelines, and the labeling for choice for consumers. Brinjal is historically a vegetable that is responsible for allergy to some people. Some media can use this information against Bt-brinjal as being allergic and toxic to both humans and animals. Currently, we did not find any strict practice of labeling to separate Bt-brinjal from non-Bt-brinjal. When both Bt-brinjal and non-Bt-brinjal are put on the market, people who would like to avoid GM food cannot exercise their right of choice.

An additional challenge associated with Bt-brinjal can result if there is a pest shift. A study in China showed that widespread adoption of Bt cotton and the associated decreased use of chemical insecticides have led to increased abundance of mirid bugs (Hemiptera: Miridae) in some fields (Lu et al., 2010). Another challenge in the sustainable use of Bt-technology is the evolution of resistance. As for other Bt-crops, over-reliance on Bt crops without appropriate Insect Resistance Management (IRM) or Integrated Pest Management (IPM) practices has led to a growing number of cases of target pest resistance (Gassmann et al., 2014; Tabashnik and Carriere, 2017). Legal court challenges

FIGURE 5 | Farmers showing their harvest of Bt-brinjal cultivated in four different study locations of Bangladesh: **(A)** Kustia Sadar, **(B)** Dinajpur Sadar, **(C)** Rajshahi Puthia, and **(D)** Pabna Sadar.

against Bt-brinjal in India and the Philippines are another controversy. However, the court case filed against Bt-eggplant in the Philippines is more of a procedural issue than a technical one. An indefinite moratorium on Bt-brinjal for mass production in India is another challenge in Bangladesh. Once the court challenges against Bt-brinjal in India and the Philippines are solved, Bt-brinjal will quickly be popularized in Bangladesh.

The present study found very significant findings. The stakeholders, expressed their satisfaction with the performance of Bt-brinjal to a considerable level. The farmers reported that cultivation of Bt-brinjal improved insect control, reduced labor and chemical costs and increased yield and income. They are happy with quality brinjal at a lower price. Reduction in pesticide application and consequently, the reduced concern of insecticide use gave an impression to the farmers that Bt-brinjal is safe for human health. However, the study revealed a limited weakness in awareness, understanding and training among the farmers on Bt-brinjal cultivation and biosafety management and also labeling of GM product. Although different government agencies arranged the training on Bt-brinjal cultivation and biosafety management system, it was not sufficient.

Moreover, some farmers are reluctant to follow the instructions properly. Lack of supervision might be another cause behind the inadequacy of biosafety management by the farmers. In a country like Bangladesh, the marketing of vegetables lacks a proper labeling of the products, especially in the local village market. The lack of appropriate labeling system during the marketing of vegetables might have caused the absence of adequate labeling of Bt-brinjal during the wholesale and retail marketing of brinjal.

CONCLUSION

Bt-brinjal is the first GM crop in Bangladesh. Some other GM crops are coming shortly. The success of Bt-brinjal cultivation can play an important role in the future of modern biotechnology in Bangladesh. The success in insect control, socioeconomic benefits to the farmers, and protection of environment, human and animal health of this first crop have set the stage for others to come. Fortunately, Bt-brinjal has a good start with increased yearly adoption and very favorable socioeconomic benefits. However, all farmers are not adequately aware of biosafety management practices and labelling of the GM brinjal is not done properly during selling them. Cultivation of Bt-brinjal facilitated control of insect, decreased insecticide use and increased yield. The reduction of pesticide application in Bt-brinjal gave farmers satisfaction. Monitoring and enforcement of the biosafety authority is also inadequate and needs need to be strengthened.

RECOMMENDATION

Bt-brinjal is a genetically modified food crop. It is the first GM crop being cultivated in Bangladesh. The stakeholders are satisfied with the Bt-brinjal to a considerable level. The further development of modern biotechnology, development and cultivation of more GM crops to face the adverse effect climate change and the challenges to feed the increasing population of the country depend on the success of Bt-brinjal cultivation in the country. The survey revealed that labeling of the Bt-brinjal during placing into the market is not done properly which is needed to inform the consumer about the product as transgenic origin. To harvest the benefits of modern biotechnology, proper management of the biosafety and labeling of the product during marketing are highly recommended. Emphasis should be given on further training of the farmers, and supervision of the appropriate authority need to be strengthened towards ensuring management of pest resistance, border crop management practice and labeling of the product in the market. Further studies covering all the districts, farmers and consumers are recommended to establish a broader picture of the Biosafety measures adopted by the farmers on Bt-brinjal in Bangladesh.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

Written informed consent was obtained from the individual(s) and/or minor(s)' legal guardian/next of kin for the publication of the identifiable images in this article.

AUTHOR CONTRIBUTIONS

Both the authors have contributions in the planning, surveying, and execution of the study. The authors contributed to the drafting and revision of the manuscript and subsequently approved the same for submission.

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Impact of Bt Brinjal Cultivation in the Market Value Chain in Five Districts of Bangladesh

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Eggplant (brinjal) is a popular vegetable that provides an important source of income for small, resource-poor Bangladeshi farmers. The biggest constraint to brinjal production is the eggplant fruit and shoot borer (EFSB). This study was conducted in 2019 in five districts in Bangladesh and examined the impacts of using genetically engineered, insect-resistant brinjal (Bt brinjal) on its value and marketing. Based on a survey of Bt and non-Bt farmers, results indicate that Bt brinjal provided an average of 19.6% higher yield and 21.7% higher revenue compared to non-Bt varieties. On a per tonne basis, the revenue benefit of using Bt brinjal was 1.7%, reflecting different levels of acceptability among trade buyers and consumers. Some were prepared to pay higher prices for Bt brinjal compared to non-Bt brinjal because the fruit was less damaged, while others paid a price discount because the Bt brinjal was not available in preferred local varieties. Labor use, expressed in 8-h days, for harvesting, grading, and packaging of Bt brinjal was 14% higher for Bt brinjal, reflecting the increased yields of Bt brinjal. 83.1% of Bt brinjal growers were satisfied with the yields obtained, and 80.6% were satisfied with the quality of fruit. This contrasts with non-Bt brinjal growers where 58.7% were satisfied with their yields and 28% indicated that a large portion of their fruit was infested, not a concern for Bt brinjal. Three-quarters of Bt brinjal growers planned to plant Bt brinjal next season because of the apparent benefits achieved of higher yields, revenue and fruit quality. Many also highlighted the benefits of reduced insecticides. Of the non-Bt growers, 39.6% had not heard of Bt brinjal. However, after hearing more about the impact of the technology, 71.4% of them indicated they planned to grow Bt brinjal next season. These findings suggest there are significant benefits of Bt brinjal and highlight the importance of making the technology available in more varieties that are suitable to local conditions and consumer preferences. Additional studies are warranted to corroborate these findings and explore in more detail the factors influencing decisions made by farmers and consumers regarding Bt brinjal.

Keywords: brinjal, eggplant, Bt, Bangladesh, value

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INTRODUCTION

Brinjal, or eggplant (*Solanum melongena* L.), is the second most important vegetable grown in Bangladesh, by about 150,000 resource-poor farmers on 50,955 hectares with a total production of 507,000 metric tonnes in 2018 (Bangladesh Bureau of Statistics (BBS), 2018). Brinjal accounted for 4.7 and 9.6%, respectively, of all winter and summer vegetable production in 2018 (Bangladesh Bureau of Statistics (BBS), 2018). Brinjal is grown in almost all agro-climatic zones with over 100 different varieties under cultivation, offering fruits of different color, size, shape, and taste. Brinjal is seriously affected by insect infestations, primarily the eggplant fruit and shoot borer (EFSB), *Leucinodes orbonalis* Guenée (Lepidoptera: Crambidae). EFSB causes between 30 and 60% yield loss, even when the crop is frequently sprayed with insecticides (Mondal and Akter, 2018). EFSB larvae damage the eggplant shoots and flowers, although the most serious damage is caused by their boring into the fruit and rendering it unmarketable. Brinjal crops are typically sprayed with insecticides over 80 times in a season of 4–5 months in all of the major growing areas in Bangladesh (Meherunnahar and Paul, 2009). This frequent application of insecticides results in very high pesticide residue levels on the fruit, kills beneficial insects, exposes farm workers to hazards, and contributes to polluting the local environment (Rahman, 2013).

Genetically engineered, insect resistant brinjal with the *cry1Ac* gene (Bt brinjal) was developed by the India-based Maharashtra Hybrid Seed Company (Mahyco) to provide an effective control of EFSB. The Agricultural Biotechnology Support Project II at Cornell University, supported by the United States Agency for International Development, facilitated the transfer of the Bt brinjal event ("EE-1") to the Bangladesh Agricultural Research Institute (BARI) and this event was introgressed into several local and commercially popular open-pollinated brinjal varieties (Shelton et al., 2018). The resulting nine Bt varieties underwent 7 years of greenhouse and confined field trials by BARI in various geographic locations in Bangladesh to test their efficacy and environmental safety. Out of those nine Bt varieties, four were subsequently approved for cultivation by the National Committee on Biosafety (NCB) of Bangladesh on October 2013. The released Bt varieties are BARI Bt Begun-1, BARI Bt Begun-2, BARI Bt Begun-3, and BARI Bt Begun-4 which are Bt isolines of Uttara, Nayantara, Kazla, and ISD006, respectively (Shelton et al., 2018). In this report they are referred to as Bt brinjal-1, Bt brinjal-2, Bt brinjal-4, and Bt brinjal-4.

These four Bt varieties are open-pollinated, which allows farmers to save seed for re-use. However, farmers are discouraged from using saved seed for multiple seasons because of potential outcrossing to other varieties, especially to non-Bt brinjal that are used in border rows as part of a refuge in a resistance management strategy (Shelton et al., 2019). After approval, the government supplied Bt brinjal seedlings to 20 selected farmers in four districts for cultivation in 2014, entrusting BARI personnel to provide training, guidance, and supervision on crop management to farmers. Since 2014, the adoption of Bt brinjal has been rapid (**Table 1**). Farmers now obtain their seed from three different Bangladeshi organizations: BARI, Department of Agricultural Extension (DAE) and the Bangladesh Agricultural Development Corporation (BADC) with seed distributed for free, except for a small charge (<US $0.10 per gram, equal to 8 Bangladesh Taka (BDT) local currency) if sourced from BADC. In 2018–9, Bt brinjal was grown by 20,695 farmers on 1,213.3 ha, equal to nearly 2.5% of the total crop (**Table 1**).

Several studies have documented the performance of Bt brinjal. In a study conducted by BARI scientists in 35 districts during the 2016–17 cropping season with 505 Bt brinjal farmers and 350 non-Bt brinjal farmers, net returns/ha, were US$2,151/ha for Bt brinjal as compared to US$357/ha for non-Bt brinjal, a 6-fold difference (Rashid et al., 2018). This study also identified that farmers spent 61% less on pesticides compared to non-Bt brinjal farmers and experienced no yield losses due to the EFSB. In a 2-year study conducted by Prodhan et al. (2018), all four Bt brinjal varieties provided virtually complete control of EFSB without the use of insecticides for EFSB control, and had higher gross returns than their non-Bt equivalents. A report by Ahmed et al. (2019) evaluated the impacts of the Bt brinjal technology on production systems, marketability, and human health. The study compared results of 600 Bt brinjal farmers and 600 non-Bt brinjal farmers living in 200 villages in four districts in the northwest of Bangladesh during the winter season of 2017-18. The results demonstrated that Bt brinjal farmers experienced significantly lower pesticide use, a reduction in overall production costs, increased yields, and provided higher profits. However, the study only included one of the four commercialized Bt varieties.

The overall objective of the present study was to identify the impact of using the four Bt brinjal varieties on the market value of the crop relative to the market value of conventional, non-Bt brinjal varieties. The specific objectives were: (1) assess the impact on the revenue generation in the value chain; (2) assess the labor use impact; (3) identify preferences and perceptions toward Bt brinjal among the farmers, traders, and consumers.

MATERIALS AND METHODS

Study Areas

Five important brinjal producing districts in Bangladesh were selected: Rangpur, Bogra, Rajshahi, Jessore, and Tangail. Within each district, one upazila (subdistrict) was randomly selected for farmer interviews, resulting in a study area of five upazilas across the five districts.

Sample Size

In each upazila, subsets of Bt brinjal and non-Bt brinjal farmers were randomly selected. The original plan was to collect data from a total of 500 farms, 250 Bt, and 250 conventional farms. However, after discarding incomplete survey responses, the total numbers of useable interview responses were 195 Bt farmers and 196 non-Bt farmers. Farmers chose to grow either a Bt brinjal variety or a non-Bt brinjal variety on their own.

Data Collection and Presentation

Face to face interviews were conducted between February and May 2019, following predesigned and pretested questionnaires. Each set of questionnaires was divided into three parts:

TABLE 1 | Farmer adoption of Bt brinjal in Bangladesh by source of seed: BARI (Bangladesh Agricultural Research Institute); DAE (Department of Agricultural Extension); BADC (Bangladesh Agricultural Development Corporation). Figures do not include farmer-saved seed.

Year	Number of farmers				Area in production (ha)			
	BARI	DAE	BADC	Total	BARI	DAE	BADC	Total
2013–14	20	0	0	20	2.83	0	0	2.8
2014–15	108	0	0	108	14.6	0	0	14.6
2015–16	250	0	0	250	10.1	0	0	10.1
2016–17	512	6,000	0	6,512	20.6	485.6	0	506.3
2017–18	581	7,601	19,430	27,612	38.9	567.8	786.3	1,392.9
2018–19	225	7,070	13,400	20,695	15.0	656.0	542.3	1,213.3

Source: USAID Feed the Future South Asia Eggplant Improvement Partnership Project, 2019.

Part 1: General information about each farmer's enterprise that was collected before harvesting.

Part II: Information about harvesting and marketing of brinjal that was collected during harvesting and the subsequent marketing period.

Part III: Post-harvest qualitative views on perceptions of Bt and non-Bt brinjal were collected after completion of harvesting and marketing.

Data are presented as mean values without deeper statistical analysis. The sample sizes and complexity of factors involved limited more detailed analysis, but the means are indicative of trends that can be followed up with more detailed studies.

Data on the monetary value are presented in local currency, the Bangladesh Taka (BDT) where 1$US equals 84 BDT.

RESULTS

Quantitative Impacts Collected Before Harvesting

In terms of age and sex distribution, primary and secondary occupation, average household size and average area of cultivated land, Bt brinjal farmers appeared not notably different from non-Bt farmers. However, Bt farmers owned 9% more land (0.83 vs. 0.76 ha) and had an 8% higher overall annual farm income (BDT 192,190 vs. 177,406). The land devoted to Bt and non-Bt brinjal cultivation varied by district (**Table 2**). Over all districts, the survey revealed slightly larger fields grown to non-Bt brinjal compared to Bt brinjal (0.08 vs. 0.07 ha). Bt farmers obtained advice to grow Bt brinjal primarily from BARI (63.3%) and DAE (33.3%). Non-Bt brinjal farmers used their traditional knowledge of brinjal production.

Quantitative Impacts Collected During Harvesting and Marketing Periods

Harvesting and Yield of Brinjal

Brinjal enters the marketing chain immediately after harvesting with farmers generally harvesting fruits 2–3 times a week during the harvesting season. The survey identified that the total number of harvests ranged from 24 to 32, occurring twice a week during the peak production period. Local traders commonly visit farmers' fields to buy fruit in bulk which they then sell at local markets either to the large wholesale traders or direct to consumers. The larger wholesale traders also procure fruits from farmers directly if they visit local markets. They then sell the brinjal at wholesale markets in the cities but this requires transportation and results in a time lag of 6–12 h before sale of the fruits in these urban markets.

The average number of harvests of fruits was the same (27.4) for both Bt and non-Bt brinjal farmers, though there was some variation between districts (**Table 3**). Overall, cultivation of Bt brinjal had no apparent impact on the frequency and number of harvests.

The average yield of Bt brinjal varieties/ha was 19.8 tonnes compared to 16.55 tonnes/ha for non-Bt brinjal varieties, indicating a 19.6% higher yield of Bt brinjal. The highest yield difference (+22.9%) was observed in Bogra, with the lowest yield difference in Jessore (+ 14.5%).

Selection of Varieties and Their Yield per Hectare

In each district, farmers typically plant varieties most suited to the local conditions and markets. Some of the Bt varieties differed from the preferred local varieties. Hence, preferences varied by district. Bt brinjal varieties 4, 3, and 2 were grown in Jessore, varieties 3 and 2 were grown in Tangail, varieties 4, 3, and 1 were grown in Bogra, varieties 4, 3, 2, and 1 were grown in Rangpur and only variety 4 was grown in Rajshahi district (**Table 4**). Our data suggest there were large differences in the yield/ha of the same Bt variety across districts. For example, the yield of Bt brinjal 4 was 17.6 tonnes/ha in Jessore, whereas it was 23.3 tonnes/ha in Bogra, 20.9 tonnes/ha in Rajshahi, and 20.3 tonnes/ha in Rangpur. Similarly, the average yield of Bt brinjal 3 was 20.8 tonnes/ha in Jessore, while it was only 17.5 tonnes/ha in Tangail. These yield differences suggest that all of the Bt varieties were not equally suitable for local growing conditions or commercially attractive enough to farmers in each district.

Average Gross Revenue Per Hectare

Within the study area, our data suggest that Bt brinjal varieties always earned higher revenue/ha than the non-Bt brinjal varieties (**Table 5**). The average gross revenue after selling Bt brinjal was estimated at BDT 312,478/ha (about $US 3,720) compared to BDT 256,718/ha (about $US 3,056) for non-Bt brinjal, a 21.7% higher revenue for the Bt varieties. The highest revenue increase (+30.2%) was observed in the Rajshahi district, while the lowest (+15.3%) increase was observed in Tangail. In terms

TABLE 2 | Average field size in hectares (ha) by district under Bt and non-Bt brinjal cultivation in the survey, 2019.

District	Jessore		Tangail		Bogra		Rajshahi		Rangpur		All Districts	
	Mean	*N*	Mean	*N*	Mean	*N*	Mean	*N*	Mean	*N*	Mean	*N*
Bt brinjal	0.06	33	0.09	40	0.07	50	0.07	37	0.05	35	0.07	195
Non-Bt brinjal	0.11	32	0.11	39	0.07	50	0.08	38	0.05	37	0.08	196

TABLE 3 | Average number of harvests and yield per hectare by district for Bt and non-Bt brinjal, 2019.

District	No. of Harvests		Yield per hectare (1,000 kg)		Yield difference (%)
	Bt brinjal	Non-Bt brinjal	Bt brinjal	Non-Bt brinjal	
Jessore	26.8	27.6	18.35	16.02	14.5
Tangail	25.0	27.8	18.12	15.69	15.5
Bogra	27.4	25.3	21.23	17.28	22.9
Rajshahi	28.7	27.8	20.91	17.27	21.1
Rangpur	29.3	29.3	20.17	17.47	15.4
All Districts	27.4	27.4	19.80	16.55	19.6

TABLE 4 | Average yield (1,000 kg per ha) of Bt brinjal varieties by district, 2019.

Bt brinjal varieties	Jessore	Tangail	Bogra	Rajshahi	Rangpur	All Districts
BARI Bt brinjal-4	17.6		23.3	20.9	20.3	20.7
BARI Bt brinjal-3	20.8	17.5	21.1		19.5	18.2
BARI Bt brinjal-2	20.1	18.5			21.8	18.7
BARI Bt brinjal-1			19.6		19.2	19.5
All Bt varieties	18.4	18.1	21.2	20.9	20.2	19.8

of returns/tonne of brinjal, the average revenue for Bt brinjal was BDT 15,769/tonne, or 1.7% higher than the revenue for non-Bt brinjal.

Differences in revenue/tonne between Bt and non-Bt brinjals varied widely across the districts. In Rajshahi, Bt brinjal earned 7.5% higher revenue than non-Bt brinjal, while the revenue/tonne advantage of Bt brinjal was only 0.7% higher in Jessore and 0.1% lower in Tangail. This difference appeared to be due to the wide price difference between Bt and non-Bt brinjal in Jessore and Tangail and reflects differences in the acceptability of the available Bt brinjal varieties vs. traditional non-Bt brinjal varieties to buyers, traders, and consumers. The level of acceptability among buyers of the Bt varieties appeared to be much higher in Rajshahi, Bogra, and Rangpur compared to Jessore and Tangail where buyers seemed to prefer the local (non-Bt) varieties and hence were prepared to pay a higher price than for the Bt brinjal.

Price, Utilization, and Revenue at Different Levels of the Market

The price of brinjal varies by the nature of the market in which it is sold. For the purpose of this study, home-consumed brinjal was assumed to be traded at the same price offered by local traders at farmers' fields. Unsold produce that remained after the end of formal sales in local markets was valued at zero if fed to cows, or assumed to be sold in lots to local traders/consumers at half of the market price. Therefore, the average value (price) attributed to the unsold element of produce (see **Table 6**) was weighted according to the volumes sold in lots at the end of market days (at 50% of the market price) and the volume fed to cows (assumed to have no value).

The data suggest the prices of Bt brinjal sold at the local markets either to wholesalers or direct to consumers (retail sales) were higher than the average price of non-Bt brinjal, although for on-farm sales to wholesalers, the price paid for non-Bt brinjal was slightly higher (**Table 6**). The average price of Bt brinjal across all markets and uses was BDT 15.78/kg compared to BDT 15.51/kg for non-Bt brinjal.

The majority of all harvested fruits was sold in the local markets via wholesales to traders. Nearly three-quarters (74.6%) of the non-Bt brinjal fruit was sold in this way, compared to about 58.2% of the Bt brinjal. The next most important outlets for Bt brinjal were retail sales to end consumers (20.2% of sales) and on-farm sales to traders (16.3% of sales). In contrast, only 6.7% of non-Bt brinjal sales were on-farm sales to traders and only 7.7% of Bt brinjal sales were to end-users (retail sales). Home consumption levels of fruit were similar for both types of fruit (about 2%), although the level of home consumption was slightly higher (0.05%) for non-Bt farmers. The unsold proportion of marketed brinjal (used as animal feed or sold off at the end of the

TABLE 5 | Average gross revenue for Bt and non-Bt brinjal in BDT*, 2019.

Districts	Per hectare		Per 1,000 kg		Revenue Increase (%)	
	Bt brinjal	Non-Bt brinjal	Bt brinjal	Non-Bt brinjal	Per hectare	Per 1,000 kg
Jessore	294,985	255,701	16,073	15,960	15.4	0.7
Tangail	285,500	247,540	15,753	15,771	15.3	−0.1
Bogra	335,941	263,627	15,827	15,257	27.4	3.7
Rajshahi	344,535	264,663	16,474	15,323	30.2	7.5
Rangpur	306,645	254,519	15,201	14,570	20.5	4.3
All Districts	312,478	256,718	15,769	15,510	21.7	1.7

*US$1 = BDT 84.

TABLE 6 | How the product was segmented in the market, its price and the revenue generated for Bt and non-Bt brinjal at different levels of the market, 2019.

How product was segmented based on percentage of the total product

Level of Market	Bt brinjal (%)	Non-Bt brinjal (%)
Home consumption	1.7	2.3
On-farm sales to traders	16.3	6.7
Wholesale at local market	58.2	74.6
Retail sale at local market	20.2	7.7
Unsold product (disposal)	3.6	8.7
Total	100.0	100.0

Price in BDT*

	Bt brinjal per kg	Non-Bt brinjal per kg
Home consumption	14.48	14.65
On-farm wholesale	14.48	14.65
Wholesale at local market	16.43	16.09
Retail sale at local market	16.94	15.29
Unsold product (disposal)	7.90	7.27
All	15.78	15.51

Revenue generated by selling in percentage of the total value of fruits

Level of Market	Bt brinjal (%)	Non-Bt brinjal (%)
Home consumption	1.6	2.2
On-farm sales to traders	15.0	6.3
Wholesale at local market	60.5	78.4
Retail sales at local market	21.7	8.5
Unsold product (disposal)	1.8	4.6
Total	100.0	100.0

*US$1 = BDT 84.

market day) was higher for non-Bt brinjal (8.7% of sales/uses) compared to the 3.6% for Bt brinjal. These data suggest that local traders and consumers preferred Bt brinjal to non-Bt brinjal, presumably because the fruits were less damaged by the EFSB.

Largely reflecting the proportion of brinjal sold in different markets, the total revenue earned from the sale of brinjal to wholesales in local markets was highest, with 60.5% of all Bt brinjal revenue coming from this sales channel and 78.4% of the non-Bt brinjal revenue coming from this sales channel. The next most important sales channels, in terms of revenue generation for Bt brinjal farmers, were retail sales to consumers which accounted for 21.7% of total revenue and on-farm sales to traders which generated 15% of total revenue. This contrasts with the non-Bt brinjal, where these two sales channels were responsible for much smaller shares of total revenue generation (8.5% for retail sales and 6.3% for on-farm sales to traders). These data suggest that while local traders preferred Bt brinjal to non-Bt brinjal for selling in local markets, non-Bt brinjals were the preferred product for traders selling in city markets. This preference for non-Bt brinjal for sale in city markets apparently was due to the non-Bt brinjal being better able to retain its skin color and texture than the Bt brinjal after 6–12 h of transportation time to city wholesale markets. Such skin color and texture are a reflection of the variety and not whether it is Bt or non-Bt.

Labor and Wages for Harvesting, Grading, and Packaging

The data suggest there was a notable employment impact associated with Bt brinjal production due to the increased yield of the marketable product (**Table 7**). Across all districts, the labor required/ha for harvesting, grading and packaging of Bt brinjal was estimated at 113.1 days (8-h day) compared to 99 days for non-Bt brinjal. An additional 21.8 days/ha were required for Bt brinjal farmers in the Jessore district compared to the non-Bt brinjal farmers. In contrast, in Tangail Bt farmers employed 1.8 fewer days for these activities than the non-Bt growers.

Harvesting, grading, and packaging are most commonly done in the early part of the day and completed in a few hours (not requiring a whole 8-h working day). This makes such work more suitable for family labor than needing to hire external labor. Out of the 14.1 full days of additional labor/ha used by Bt brinjal farmers compared to non-Bt farmers, 9.2 days were family labor and 4.9 were hired labor (60 and 40%, respectively).

Total wages paid to hired labor for harvesting, grading, and packaging were BDT 17,829/ha for Bt brinjal as compared to BDT 17,099 for non-Bt brinjal. However, in terms of harvesting labor costs/tonne of produce, the hired labor cost was lower for

TABLE 7 | Labor and wages for harvesting, grading, and packaging Bt and non-Bt brinjal by district, 2019.

Labor in 8-hour days

Districts	Bt brinjal (days)			Non-Bt brinjal (days)		
	Family	Hired	Total	Family	Hired	Total
Jessore	75.6	46.2	121.8	57.6	42.5	100.0
Tangail	59.0	41.8	100.8	57.8	44.8	102.6
Bogra	67.2	46.2	113.4	67.2	43.9	111.1
Rajshahi	73.8	44.4	118.2	59.0	37.0	95.9
Rangpur	71.5	44.7	116.2	68.6	44.1	112.7
All Districts	68.4	44.7	113.1	59.2	39.8	99.0

Wages paid for harvesting, grading and packaging in BDT*

Districts	Wage per hectare		Wage per 1,000 kg	
	Bt brinjal	Non-Bt brinjal	Bt brinjal	Non-Bt brinjal
Jessore	17,897	16,459	967	1,027
Tangail	16,844	16,958	929	1,081
Bogra	18,655	17,277	879	1,000
Rajshahi	18,699	17,719	934	1,026
Rangpur	16,222	16,517	799	946
All Districts	17,829	17,099	918	1,033

**US$1 = BDT 84.*

TABLE 8 | Marketing cost for Bt and non-Bt brinjal by district in BDT*, 2019.

District	Cost per hectare		Cost per 1,000 kg	
	Bt brinjal	Non-Bt brinjal	Bt brinjal	Non-Bt brinjal
Jessore	24,599	20,593	1,287	1,285
Tangail	22,360	18,213	1,219	1,179
Bogra	24,129	20,858	1,213	1,207
Rajshahi	21,692	18,741	1,117	1,085
Rangpur	26,025	21,684	1,305	1,241
All Districts	23,677	19,826	1,227	1,203

**US$1 = BDT 84.*

Bt brinjal farmers (BDT 918) compared to non-Bt brinjal farmers (BDT 1033).

Marketing Costs

Marketing costs/ha, including costs of transportation from farm to market and market tolls, appeared to be higher for Bt brinjal (BDT 23,677/ha of crop) compared to BDT 19,826 for non-Bt brinjal (**Table 8**). However, the marketing cost of Bt brinjal/tonne was similar to the cost of non-Bt brinjal. These differences appear to reflect the yield differences between the two crops.

Qualitative Impacts Collected After Completion of Harvesting and Marketing

In the survey, additional questions were asked of the farmers about their current knowledge and experience with the Bt brinjal relative to local non-Bt brinjal varieties in order to assess reasons for adoption and prospects for future use of the technology.

Overall Satisfaction With the Bt Brinjal

Over all districts, 80.6% of the Bt brinjal farmers, compared to 71.9% of non-Bt brinjal farmers, appeared to be satisfied with the quality of their respective produce (**Table 9**). A larger proportion of Bt farmers (83.1%) were satisfied with the fruit yield/ha compared to yield satisfaction levels of non-Bt farmers (58.7%). Only in Jessore and Tangail were there any farmers expressing less satisfaction with Bt brinjal than non-Bt brinjal.

Problems Encountered With Growing, Selling, or Marketing Produce

For the 19.4% of Bt brinjal growers that expressed some concern with their crop, the main concern related to skin of the brinjal (14.9%) that adversely affected the product quality during transportation from local markets to city markets (**Table 10**). In relation to concerns with the quality of non-Bt produce, of the 28.1% of farmers who expressed some concern, the main concern was insect infestation in a large portion of their harvested fruits (26.5%).

Overall, 56.9% of Bt farmers and 48.5% of non-Bt farmers indicated that they had faced problems selling their produce. The most popular complaint of the Bt brinjal farmers who had experienced problems was that the price received was lower than the price of local and popular non-Bt varieties (37.9%), although this perception is inconsistent with the actual gross revenue data presented in **Table 5**.

Overall, 36.9% of the Bt brinjal growers perceived that traders were not interested in buying Bt brinjal and 28.7% of these

TABLE 9 | Grower satisfaction with the quality of fruit and yield of Bt and non-Bt brinjal by district, 2019.

Districts	% satisfied with quality		% satisfied with yield	
	Bt brinjal	Non-Bt brinjal	Bt brinjal	Non-Bt brinjal
Jessore	66.7	84.4	53.5	81.3
Tangail	54.5	100.0	65.0	87.2
Bogra	96.0	40.0	98.0	46.0
Rajshahi	91.9	73.7	97.3	26.3
Rangpur	95.0	73.0	100.0	59.6
All Districts	80.6	71.9	83.1	58.7

Figures represent the percentage of the total number of farmers.

TABLE 10 | Grower concerns with growing, selling, and marketing Bt brinjal and non-Bt brinjal by district, 2019.

Growing

Quality concerns	Jessore	Tangail	Bogra	Rajshahi	Rangpur	All Districts
Bt brinjal						
Color, shape and size of fruit not attractive	18.2	20.0	0	0	0	7.2
Tough fruit	0	10.0	4.0	0	0	3.1
Skin affected during transportation	30.3	35.0	0	8.2	5.0	14.9
No comments	9.1	2.5	0	0	0	2.1
Farmers expressing concerns (%)	33.3	45.5	4.0	8.1	5.0	19.4
Non-Bt brinjal						
A large portion of the harvest was infested	15.6	0	54.0	26.3	27.0	26.5
Farmers expressing concerns (%)	15.6	0	60.0	26.3	27.0	28.1

Selling

Districts	Bt brinjal		Non- Bt brinjal	
	Faced problems	Did not face problems	Faced Problems	Did not face problems
Jessore	93.9	6.1	34.4	65.6
Tangail	80.0	20.0	30.8	69.2
Bogra	22.0	78.0	48.0	52.0
Rajshahi	16.2	83.8	73.7	26.3
Rangpur	29.1	70.9	54.1	45.9
All Districts	56.9	43.1	48.5	51.5

Marketing

Problems	Jessore	Tangail	Bogra	Rajshahi	Rangpur	All Districts
Bt brinjal						
Price of Bt brinjal was lower than non- Bt brinjal	93.9	80.0	10.0	16.2	0.0	37.9
Traders are less interested to buy Bt brinjal	84.8	72.5	12.0	16.2	8.6	36.9
Consumers are less interested to buy	81.8	42.5	12.0	16.2	0	28.7
Traders/consumers had negative perception	54.5	42.5	0	2.7	8.6	20.0
Color and shape was not like the local brinjal	9.1	15.0	0	0	0	4.6
Farmers facing problems (%)	93.9	80.0	22.0	16.2	29.1	56.9
Non-Bt brinjal						
Did not get expected price as the fruits were infested	18.8	30.8	48.0	73.7	54.1	45.9
A large amount remained unsold	3.1	0	12.0	2.6	0	4.1
Farmers facing problems (%)	34.4	30.8	48.0	73.7	54.1	48.5

Figures represent the percentage of farmers with concerns.

farmers also perceived that consumers were not interested in buying Bt brinjal. As highlighted above relating to perceptions relating to difficulties selling Bt brinjal, these perceptions about trader and consumer purchasing preferences appeared to be inconsistent with the volumes left unsold (see below).

Outcome of Marketing Bt and Non-Bt Brinjal

Of the Bt brinjal farmers, there was considerable variation between districts but, over all districts, 21.0% reported that a large portion of their produce remained unsold and 29.2% indicated that they had to sell their produce at below a perceived market price (**Table 11**). However, over all districts, 27.2% of the Bt brinjal farmers did not perceive they suffered a loss. Among the non- Bt farmers, 45.9% thought they sold their produce below a perceived market price and 6.1% reported that a large portion of their produce remained unsold. Farmer complaints about selling under a perceived market price are common across commodities.

Factors Influencing the Decision to Cultivate or Not Cultivate Bt Brinjal

Over all districts, 88.7% of Bt brinjal farmers chose these varieties because they believed that infestation levels of EFSB would be minimal and insecticide cost would be notably lower than if they grew non-Bt brinjal varieties (**Table 12**). In addition, 70.3% of Bt brinjal growers anticipated higher yields than if they grew non-Bt brinjal.

It is interesting to note that an average of 39.6% (range of 18.0–66.7%) of the non-Bt brinjal farmers over all the districts were unaware about Bt brinjal technology. This suggests the need for focusing educational efforts on specific districts where farmers are unaware of Bt brinjal. Of those farmers who were aware of Bt brinjal (59.4% of non-Bt growers), 42.1% thought that Bt brinjal would have a lower market price than non-Bt brinjal and 14.2% thought it would have a lower yield. As the findings summarized in **Table 3**, **Table 11** show, these perceptions appear to be incorrect.

Awareness of Negative Information About Bt

For both Bt and non-Bt brinjal farmers, about 80% were not aware of any negative information against Bt brinjal (**Table 13**). Of those who were aware of negative information (20% of the total), the main negative information they were aware of (for both Bt growers and non-Bt growers) related to the perception that Bt brinjal was not safe for human consumption or the environment. This finding should be addressed in future educational programs.

Perceptions of Non-Bt Brinjal Farmers About Growing Bt Brinjal

Over three-quarters (75.5%) of the non-Bt brinjal farmers had heard opinions from neighboring farmers who were cultivating Bt brinjal (**Table 14**). About half (49.5%) of them heard from neighboring Bt brinjal farmers that growing Bt brinjal was a good decision. The main positive experiences heard were that Bt farmers applied less insecticides and this improved their health and environment (51.0%), and that Bt brinjal was more profitable (43.4%). This important finding should be explored with additional studies.

Decisions by Bt and Non-Bt Farmers to Grow Bt Brinjal Next Year

When asked just after the harvest about future plans to grow Bt brinjal, 75.4% of the current year's Bt brinjal farmers and 71.4% of the current year's non-Bt farmers stated that they planned to grow Bt brinjal in the upcoming crop season (2020) (**Table 15**). Some Bt brinjal growers indicated that they would not plant Bt brinjal next season. The highest percentage of Bt brinjal farmers who stated they would not grow Bt brinjal next year were farmers from Jessore (48.5%) and Tangail (70.0%). These were the districts where they had concerns about selling their crop (**Table 11**). The non-Bt farmers who planned not to grow Bt brinjal the next season perceived there would not be high demand for Bt brinjal (8.7%) and that the low cost of insecticides would allow them to control EFSB (10.7%). Only 6.6% perceived health and environmental benefits. These findings warrant further studies on these issues.

Of the current year's non-Bt farmers who planned to grow Bt brinjal next season, the main reasons for doing so were their perceptions that yield of Bt brinjal would likely be higher than non-Bt brinjal (22.4%), there would be less damage by EFSB infestation (20.4%), and higher profitability (13.8%) (**Table 15**). Overall, these views suggest that farmers who have grown Bt brinjal largely perceive the technology has delivered benefits, but those who have not grown Bt brinjal remain to be convinced of its potential benefits.

DISCUSSION

The overall objective of this research was to study the impact of Bt brinjal in the market value chain relative to locally popular non-Bt brinjal varieties, with respect to income and employment generation, and assess preference factors and perceptions about Bt brinjal that farmers had, and what they perceived the views of traders and consumers were about the product. Because of the many factors explored in this survey, analysis of each was limited to presenting results as the means of the values. Mean values provide indications of differences between the treatments and are commonly used in such agronomic surveys (e.g., Gusta et al., 2011; Hudson and Richards, 2014). However, future studies that explore many of our findings should be designed with more powerful analyses.

There were few and only minor differences in the family unit and economic status of those who chose to grow Bt brinjal or non-Bt brinjal. Thus, it appears that such socioeconomic factors did not influence the farmer's decision to grow either type of crop and analysis could justifiably focus on the crop's performance and value.

Important findings of this study indicate a 19.6% higher average yield (**Table 3**), a 21.7% higher average gross revenue (**Table 5**) and a 1.7% average revenue generation/tonne for Bt brinjal compared to non-Bt brinjal. This additional revenue/ha is equal to about $US 664, a substantial sum for resource-poor farmers in Bangladesh. This increased revenue appears to be due to higher yields and less inputs. While we did not break out pesticide costs in this study, previous reports in Bangladesh have

TABLE 11 | Outcomes in marketing Bt brinjal and non-Bt brinjal by district, 2019.

Outcomes	Jessore	Tangail	Bogra	Rajshahi	Rangpur	All Districts
Bt brinjal						
A large portion of the product remained unsold	36.4	37.7	4.0	32.4	0	21.0
Products sold at lower than market price of brinjal	78.8	62.5	12.0	0	0	29.2
No loss as such	15.2	27.5	2.0	97.3	0	27.2
No comments	0	2.5	4.0	0	2.9	2.1
Non-Bt brinjal						
A large portion of the product remained unsold	6.3	0	6.0	13.2	5.4	6.1
Products sold at lower than market price of brinjal	28.1	30.8	56.0	60.5	48.7	45.9

Figures represent the percentage of the outcomes.

TABLE 12 | Perceptions influencing the farmers' decision to grow Bt brinjal by district, 2019.

Perceptions of Bt brinjal farmers about why they grow Bt brinjal

Perception	Jessore	Tangail	Bogra	Rajshahi	Rangpur	All Districts
Yield of Bt brinjal was higher than non-Bt brinjal	9.1	87.5	56.0	100.0	97.1	70.3
Bt brinjal was a better-quality product than non-Bt brinjal	21.2	37.5	72.0	100.0	97.1	66.2
Bt brinjal market price was higher than non-Bt brinjal	0.0	12.5	54.0	0.0	97.1	33.8
Infestation of EFSB in Bt brinjal was minimal	93.9	77.5	80.0	100.0	97.1	88.7
Insecticides costs was notably lower with Bt brinjal	93.9	97.5	76.0	100.0	97.1	91.8

Perceptions of non-Bt brinjal farmers about why they do not grow Bt brinjal

Factors	Jessore	Tangail	Bogra	Rajshahi	Rangpur	All Districts
Lower yield of Bt brinjal than non-Bt brinjal	45.5	0	24.0	0	2.7	14.2
Lower market price of Bt brinjal than non-Bt brinjal	51.5	66.7	12.0	86.8	2.7	42.1
Almost same insecticides costs	6.1	17.9	2.0	0	0	5.1
Not getting seeds or seedlings in time	12.1	0	34.0	0	0	10.7
Not safe for human and environment	0	0	0	0	21.6	4.0
Not a suitable crop to be grown in Bangladesh	0	15.4	0	0	8.1	4.6
Didn't know about Bt brinjal	66.7	59.0	18.0	31.6	32.4	39.6

Figures represent the percentage of the perception.

documented a 61% decrease in pesticide costs (Rashid et al., 2018) while Ahmed et al. (2019) reported that Bt brinjal farmers spent BDT 7,174 less on pesticides/ha compared to control farmers.

Another important suggestion from this study is the variable performance of the Bt brinjal varieties in different districts relative to local varieties. This highlights that the four Bt brinjal lines are not ideally suited for all regions, not only in terms of agronomic performance but also in terms of consumer preferences relating to the fruit. This is clearly shown by the growers who chose to grow or not grow a Bt brinjal line in a particular district, such as Bt brinjal-4 which was grown in Jessore and yielded 17.6 tonnes/ha compared to 23.3 tonnes/ha in Rajshahi, a difference of 25% (**Table 4**).

Preference for a type of brinjal appears to be a strong consideration for consumers and marketers and includes color, shape, and size. Whether the product is Bt on not, appears to be of secondary interest, although this varies across districts. Of the farmers growing Bt brinjal, 43.1% did not face any problems selling their product, compared to 51.5% of the non-Bt brinjal farmers (**Table 10**). In Rangpur and Bogra, where Bt brinjal has been extensively grown since 2014, 4% of Bt farmers had a large portion of the product unsold, while in the other districts the rates were higher and the average for all regions was 21.0% (**Table 11**). Non-Bt brinjal farmers had an overall lower portion of their product remaining unsold. However, Bt farmers stated that 27.2% of their produce was sold at a lower perceived market price for brinjal, as compared to 45.9% of non-Bt brinjal farmers.

About 75% of non-Bt brinjal fruits were sold to wholesale traders for supply to city markets as compared to 58.2% of Bt brinjal (**Table 6**). Accordingly, non-Bt brinjal farmers earned 78.4% of their revenue from sales to wholesalers compared to 60.5% for Bt farmers. The primary reason for Bt brinjal remaining unsold (when this was the case) appears to be related to the non-Bt brinjal being able to better retain its skin color and texture than the four Bt brinjal varieties after 6–12 h of transportation time to the city wholesale markets. It should be noted, however,

TABLE 13 | Awareness of negative information about Bt brinjal and the type of information by district.

Awareness of negative information

Districts	Bt brinjal farmers		Non-Bt brinjal farmers	
	Aware	Not aware	Aware	Not aware
Jessore	39.4	60.6	31.1	68.8
Tangail	5.0	95.0	25.6	74.4
Bogra	10.0	90.0	2.0	98.0
Rajshahi	0	100.0	0.0	100.0
Rangpur	57.1	42.9	48.6	51.4
All Districts	20.5	79.5	19.9	80.1

Type of negative information

Information/Issues	Bt farmers	Non-Bt farmers
Yield of Bt brinjal is not higher than non-Bt brinjal	7.7	4.6
Infestation of EFSB in Bt brinjal is almost the same as non-Bt brinjal	0.5	0.5
Bt brinjal is not safe for human consumption	14.4	11.2
Bt brinjal is not safe for the environment	12.3	13.3
Bt brinjal may affect surrounding crops including non- Bt brinjal	2.1	0.5
Bt brinjal is not a suitable crop to be grown in Bangladesh	0.5	0.0

Figures represent the percentage of the farmers, 2019.

that differences in the skin and texture are related to varietal differences and are not associated with the Bt trait. As additional Bt varieties are being developed, the shipping quality and desires of the urban consumer should be considered.

Our data indicate that labor usage and wages for harvesting, grading, and packaging were higher for Bt brinjal (an additional 14.1 days of labor required and an additional BDT 730/ ha, **Table 7**). This reflected the higher yield of Bt brinjal, a desirable trait for a farmer. This increase in labor usage and cost was probably largely offset by the reduction in labor time and cost requirements for spraying insecticides, although this aspect of labor and insecticide costs were not specifically examined in this study.

Besides the savings in pesticide costs noted above (Rashid et al., 2018; Ahmed et al., 2019), there will be labor savings for not applying insecticides. An ex-ante study by Islam and Norton (2007) estimated that insecticide labor cost would be reduced by about $34/ha with Bt brinjal, which would offset the additional costs for harvesting and packing identified in this study. This suggests the two categories effectively cancel each other out, so the overall impact on labor usage and pesticide costs would be neutral. Additionally, the Islam and Norton study (2007) estimated that the cost of insecticides would likely be reduced by $36.36/ha. A similar pattern of labor change has been observed with the adoption of other insect-resistant crops like Bt cotton in India. For example, Qaim et al. (2006) found that reduced cotton insecticide sprayings resulted in a lower requirement for labor to undertake pest scouting and spraying (this mostly affected male family members) but this was counterbalanced by additional labor requirements for harvesting the higher yielding crop, with the latter labor change mainly affecting casual, usually female labor. Overall, they concluded that the net effect on labor use was largely neutral. Later work by Subramanian and Qaim (Subramanian and Qaim, 2009) found that the use of Bt cotton in India resulted in a net increase in labor, with the additional requirement for labor (largely female) for harvesting, outweighing the decrease in requirement for insecticide spraying.

In terms of satisfaction with the Bt brinjal technology, our data suggest that most Bt brinjal farmers were satisfied with the quality (80.6%) and yield (71.9%) of their produce (**Table 9**). This compared with non-Bt brinjal growers who had a similar level of satisfaction with the quality of produce (83.1%) but a lower level of satisfaction with yield (58.7%). The level of satisfaction with the technology expressed by Bt growers can also be seen in the fact that they decided to grow Bt brinjal because 88.7% believed that infestation of EFSB would be minimal and 91.8% believed their insecticide cost would be notably lower than with non-Bt brinjal (**Table 12**). For non-Bt growers, it is important to note that 39.6% of them had no knowledge of Bt brinjal (**Table 12**). Of the non-Bt growers who had some knowledge of Bt brinjal, the most important reason given for not trying Bt brinjal was fear that the fruit would obtain a lower price, followed by a view that Bt yields would be lower than non-Bt yields. Future communication efforts should focus on increasing farmers' awareness of Bt brinjal and the increased yield and revenue it generates.

In relation to the possible influence of negative information being available about Bt technology and potentially discouraging its adoption, among both Bt and non-Bt brinjal farmers a large majority (about 80% of each type of farmer) indicated that they were not aware of any negative information about Bt brinjal (**Table 13**). For the non-Bt brinjal growers, information about the performance of Bt brinjal appears to have had a positive influence on future planting intentions because 71.4% of the non-Bt growers indicated they would grow Bt brinjal next season (**Table 14**). However, this varied by district with only 10.3% in Tangail interested in growing Bt brinjal next season. The main reasons cited for future adoption of the technology was the expectation of increased yield of Bt brinjal (22.4%) and decreased attack by EFSB (20.4%). These findings suggest additional studies are warranted on these issues.

Previous studies on the impact of using Bt brinjal (see introduction) have shown virtually complete control of EFSB in Bangladesh without any disruption of non-target arthropods (Prodhan et al., 2018). In the Philippines, a similar level of control (Hautea et al., 2016) and lack of effect on non-target arthropods (Navasero et al., 2016) was observed. In both studies, the Bt lines were directly compared to their non-Bt lines (same variety but without the Bt gene) and always showed superior performance for each Bt line compared to its non-Bt line. In the present study,

TABLE 14 | Non-Bt farmers who heard opinions from neighboring farmers about growing Bt brinjal and what those opinions were by district.

Were opinions heard?						
District	**Jessore**	**Tangail**	**Bogra**	**Rajshahi**	**Rangpur**	**All Districts**
Heard	75.0	48.7	76.0	100.0	78.4	75.5
Did not hear	25.0	51.3	24.0	0.0	21.6	24.5
What opinions were heard?						
Opinion	**Jessore**	**Tangail**	**Bogra**	**Rajshahi**	**Rangpur**	**All Districts**
Growing Bt brinjal was a good decision	34.4	2.6	44.0	100.0	67.6	49.5
Growing Bt brinjal was a wrong/bad decision	9.4	41.0	0.00	0.0	0.00	9.7
Incurred losses compared to non-Bt farmers	34.4	41.0	0.00	0.0	0.00	13.8
Made good profit compared to non-Bt farmers	9.4	2.6	36.0	100.0	67.6	43.4
The product quality was better than non-Bt farmers	37.5	0.00	26.0	100.0	46.0	40.8
Applied less insecticide and improved farmers' health and the environment	59.4	41.0	18.0	100.0	48.7	51.0
Problem in selling and demand was less in the market	9.4	30.8	0.0	0.00	0.00	7.7
Yield of Bt brinjal was higher	0.00	0.00	24.0	84.2	24.3	27.0

Figures represent the percentage of the farmers, 2019.

TABLE 15 | Decision and reasons to grow Bt brinjal the following year by districts.

Decision to grow Bt brinjal				
Districts	**Bt brinjal farmers**		**Non-Bt brinjal farmers**	
	Will grow	**Will not grow**	**Will grow**	**Will not grow**
Jessore	51.5	48.5	59.4	40.6
Tangail	30.0	70.0	10.3	89.7
Bogra	94.0	6.0	86.0	14.0
Rajshahi	100.0	0	100.0	0.0
Rangpur	97.1	2.9	97.3	2.7
All districts	75.4	24.6	71.4	28.6

Reasons expressed by the current non-Bt brinjal farmers to grow Bt brinjal the following year						
Reason	**Jessore**	**Tangail**	**Bogra**	**Rajshahi**	**Rangpur**	**All Districts**
High yield of Bt brinjal	6.3	0.0	12.0	52.6	43.2	22.4
High demand in the market	0.0	0.0	8.0	2.6	32.4	8.7
No attack by EFSB	21.9	0.0	20.0	50.0	10.8	20.4
More profitable	3.1	0.0	6.0	44.7	16.2	13.8
Low cost of insecticide	15.6	0.0	18.0	0.0	18.9	10.7
Safe and good for health and environment	12.5	0.0	0.0	0.0	24.3	6.6

Figures represent the percentage of the farmers, 2019.

many comparisons were made between two brinjal groups (Bt brinjal to non-Bt brinjal), regardless of variety background. Still the trends appeared similar in that the Bt lines provided better control of EFSB, higher yields and increased revenue.

The economic benefits of Bt brinjal are reduced use and cost of insecticides, higher yields and higher returns to farmers (see for example, Rashid et al., 2018). This study is consistent with these earlier studies and extends the analysis to better understand the impacts post-farmgate and to understand the factors that influence brinjal farmers to decide to grow or not to grow Bt brinjal. The results from the present study suggest that additional follow-up studies that focus on farmers' planting decisions and consumers' purchase decisions are warranted.

The rapid adoption of the technology between 2014 and 2019 (**Table 1**) suggests that adopters are obtaining important benefits across a wide range of regions. In addition, as most Bt brinjal growers have not experienced difficulties in selling their

produce, this suggests that the reduced levels of fruit damage make the Bt fruit more attractive to many consumers. For the future, however additional adoption of the technology will depend on availability of the Bt technology in a wider range of varieties, suitable for growing in more localities and which offer the desired characteristics of consumers. Most importantly, these findings indicate the need for an active education program for brinjal farmers since nearly 40% of them were unaware of Bt brinjal, and some had misconceptions about its safety and marketability.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article.

AUTHOR CONTRIBUTIONS

SS, AS, MH, and VP designed the study. SS performed the research. SS and AS analyzed data. AS, SS, GB, MH, and VP wrote paper.

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REVIEW
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Genetic Biocontrol for Invasive Species

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Invasive species are increasingly affecting agriculture, food, fisheries, and forestry resources throughout the world. As a result of global trade, invasive species are often introduced into new environments where they become established and cause harm to human health, agriculture, and the environment. Prevention of new introductions is a high priority for addressing the harm caused by invasive species, but unfortunately efforts to prevent new introductions do not address the economic harm that is presently manifested where invasive species have already become established. Genetic biocontrol can be defined as the release of organisms with genetic methods designed to disrupt the reproduction of invasive populations. While these methods offer the potential to control or even eradicate invasive species, there is a need to ensure that genetic biocontrol methods can be deployed in a way that minimizes potential harm to the environment. This review provides an overview of the state of genetic biocontrol, focusing on several approaches that were the subject of presentations at the Genetic Biocontrol for Invasive Species Workshop in Tarragona, Spain, March 31st, 2019, a workshop sponsored by the OECD's Co-operative Research Program on Biological Resource Management for Sustainable Agricultural Systems. The review considers four different approaches to genetic biocontrol for invasive species; sterile-release, YY Males, Trojan Female Technique, and gene drive. The different approaches will be compared with respect to the efficiency each affords as a genetic biocontrol tool, the practical utility and cost/benefits associated with implementation of the approach, and the regulatory considerations that will need to be addressed for each. The opinions expressed and arguments employed in this publication are the sole responsibility of the authors and do not necessarily reflect those of the OECD or of the governments of its Member countries.

Keywords: invasive species, genetic biocontrol, gene drive, Trojan Female Technique, Trojan Y Chromosome

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INVASIVE SPECIES

As global trade increases the transfer of goods and commodities around the world, it leads to the movement of species from their native ranges to new locations where they become invasive. According to United States Executive Order 13112 of February 3, 1999 (Invasive Species), invasive species are defined as "an alien species whose introduction does or is likely to cause economic

or environmental harm or harm to human health" (Executive Order 13112, 1999). Whether or not a species becomes invasive depends on the characteristics of both its life history traits and the environment in which it is introduced. Not all new species introductions become invasive, however, species that become invasive in a new environment can have profound effects on industry, agriculture, and conservation lands within the new location where they become established (Paini et al., 2016).

Eradication of invasive species is often impractical once they have become established (Britton et al., 2011). In some cases, it is possible to contain invasive species at the site where the initial introduction occurred and eradicate the population before it has the opportunity to spread beyond the limits of the area where eradication measures are effective (Steck et al., 2019). In other cases, eradication is not considered an option and management plans are instead implemented as a means of preventing further spread. Most control efforts primarily focus on limiting the harm associated with the invasive species to an acceptable level. In many cases this may involve integrated pest management (IPM), an approach which utilizes multiple pest management tools in the hopes of obtaining a synergistic control effect (Kogan, 1998). Although the result of such an approach can be expensive and sometimes inefficient, there are few options to effectively limit the harm associated with invasive species.

GENETIC BIOCONTROL STRATEGIES

Genetic biocontrol provides opportunities for the control and potential eradication of invasive species. The term "genetic biocontrol" refers to techniques that alter the genetic material of an organism to control invasive species in the environment. Some, but not all, of these techniques involve knowledge or manipulation of the genome. This includes utilization of naturally occurring genotypes; parasitic microbes that distort sex ratios; the use of traditional methods such as irradiation, hormonal sex reversal to generate sterile, or sexually incompatible genotypes; and the use of modern genetic engineering technologies. Because genetic biocontrol describes a wide variety of methods that take advantage of species biology in order to achieve control, it is important to note that genetic biocontrol is not a synonym for the use of genetically engineered organisms. Existing technologies that use naturally occurring genetic alleles, irradiated organisms, chromosomal segregation techniques, or endoparasitic bacteria (i.e., *Wolbachia*) constitute genetic biocontrol techniques that would not be considered genetic engineering.

This review provides an overview of the state of genetic biocontrol, focusing on several approaches that were the subject of presentations at the Genetic Biocontrol for Invasive Species Workshop in Tarragona, Spain, March 31st, 2019, an OECD Co-operative Research Programme sponsored conference. The review will highlight the range of genetic biocontrol options that are available (or are in development) for invasive species control, examining the attributes of four approaches; sterile-release, YY Males, Trojan Female Technique, and gene drive. The review compares techniques regarding the mechanism of control used in each method, and the regulatory hurdles that must be overcome for the genetic biocontrol methodology to be put into practice. Because gene drive is a fundamentally new approach to invasive species control that presents new possibilities for efficient suppression of invasive species populations (and may therefore pose new risk as well), research efforts to test containment for gene drives (both physical and genetic containment) are also reviewed. Lastly, the paper presents a review of classical biocontrol to provide context around ongoing practices for invasive species management and to highlight existing regulatory frameworks involving the release of a new organism into the environment that are likely to be relevant to the use of newer genetic biocontrol methods.

Sterile Insect Technique (SIT) as a Reference Point for Considering Genetic Biocontrol

One of the earliest applications of genetic biocontrol involved irradiation of the screw worm *Cochliomyia hominivorax* (Coquerel) as a means of producing sterile individuals that could be generated in large numbers and distributed within a target population in order to suppress reproduction. The technique, known as Sterile Insect Technique (SIT) successfully eradicated screw worms from the southeastern United States (Knipling, 1955; Smith, 1963), and has since been employed for the control of a variety of other insects [e.g., the codling moth (*Cydia pomonella* (L.)) (Bloem et al., 2007)], the pink bollworm [*Pectinophora gossypiella* (Saunders) (Tabashnik et al., 2010)], and the painted apple moth [*Teia anartoides* (Walker) (Suckling et al., 2007)]. Preliminary work on vectors of human disease such as the yellow fever mosquito [*Aedes aegypti* (L.) (Alphey et al., 2010)] and Tsetse fly [*Glossina* spp.) (Vreysen et al., 2014] has been conducted, with more work needed to achieve effective control by sterile-release.

Insects are irradiated for SIT using a dose of gamma radiation sufficient to cause chromosomal breaks within the germ line. For some insect species, SIT does not substantially alter mating competitiveness. However, there are limitations to this technique in that some species have incomplete sterilization, causing a serious decrease in competitiveness and an insufficient sterile population, amongst other issues (Esteva and Mo Yang, 2005). If it is practical to do so, males are sorted from females to allow release of only sterile males into the environment. This leads to a more efficient suppression of the target population (up to 3–5-fold) than if both sexes are released together (Rendón et al., 2004; Alphey and Bonsall, 2018). Irradiated insects are then released into the target area in sufficient numbers to ensure that fertile individuals will have a high probability of encountering irradiated sterile individuals leading to unproductive mating.

Although SIT has been a successful approach to control some insect pests, there are disadvantages associated with its use. In order to be successful in suppressing a target population, an overwhelming number of irradiated organisms must be released into the environment, thus temporarily increasing the potential

impacts caused by the pests. To produce the required numbers of sterile insects, a dedicated facility to rear and irradiate organisms for release is required, which can be costly to construct and operate. Some species are not suitable for sterile release because of limitations in rearing, thus the technique may be unsuitable for control of some species. The effects of radiation on insect mating competitiveness can also be a disadvantage, requiring that even greater numbers of irradiated insects are released in order to compensate for reduced mating efficiency.

As an alternative to SIT, genetic engineering has also been used to produce sterile insects for release to suppress a target population (Alphey and Bonsall, 2018). This technique has resulted in transgenic insects that are reproductively sterile as a result of a transgene that confers a dominant lethal phenotype to progeny that inherit it (RIDL, Release of Insects Carrying a Dominant Lethal) (Alphey, 2014). As with SIT, an overwhelming abundance of RIDL males must be released into a target population in order to ensure that wildtype females have only unproductive matings with RIDL partners and population suppression ensues (**Figure 1**). RIDL is thus an improvement over SIT with respect to practical aspects of producing insects with higher mating competitiveness but shares the same disadvantage of SIT in that many insects must be reared and released. This intrinsic disadvantage of sterile-release is also relevant to the use of this method to control non-arthropod invasive species.

Invasive bullfrogs [*Lithobates catesbeianus* (Shaw)] in Europe are not amenable to mass sterilization by radiation, therefore require an alternative strategy to produce sterile individuals for sterile-release. As demonstrated in a recent pilot study, induced triploidy can reliably produce sterile bullfrogs in sufficient numbers to eradicate a small target population under containment conditions (Descamps and De Vocht, 2017). Induced triploidy is a technique which disrupts meiosis by chemical, mechanism, or thermal methods, resulting in eggs or sperm containing three sets of chromosomes. Triploidy in reproductive cells results in abnormal development that can abort the reproductive process. However, future work on sterile-release efforts requires construction of a dedicated facility to rear sterile triploid bullfrogs in sufficient numbers for release. As adult bullfrogs can exceed 30 cm in length, the facility will need to be quite large and require substantially greater resources for care and feeding than insects. A further concern is that addition of an abundance of sterile bullfrogs to a target population will cause environmental harm. Such a program will thus require some form of manual wild bullfrog removal in an integrated pest management program to mitigate the harm associated with the introduction of additional sterile individuals. Such an approach melding population suppression and the addition of sterile individuals has been attempted with another invasive vertebrate, the Sea Lamprey in the St. Mary's River, a tributary of the Laurentia Great Lakes (Bravener and Twohey, 2016). Further harm resulting from the introduced sterile bullfrogs might be reduced if sterile individuals are added at a very early stage of development so that the sterile individuals have a smaller impact as competitors for food with the native aquatic organisms higher in the food chain.

Trade-Offs Between Efficiency, Control, and Uncertainty of SIT

Although sterile-release has been a very successful approach for the control of pest insects, radiation-induced sterility of invasive species for sterile-release has limited applicability as a genetic biocontrol strategy for most invasive species. The approach may be restricted to a subset of arthropod invasive species; those that can be reared, irradiated, and distributed without substantial negative effects on the viability and mating effectiveness of the released insects. For species not amenable to radiation-induced sterilization, other genetic approaches to sterility (e.g., induced triploidy for fish and amphibians) or the Trojan Female Technique (Gemmell et al., 2013) may offer an alternative approach to radiation for the production of sterile individuals. For insects, RIDL provides an alternative to SIT and can be applied to a variety of invasive insect species affecting agriculture (Ant et al., 2012; Leftwich et al., 2014; Alphey and Bonsall, 2018). However, the principal limitation in using sterile-release as a general approach for invasive species control is the problem of limiting the harm associated with the large number of sterile individuals required to suppress the fertility of the target population. In some cases, harm is specific to one sex only and can be avoided by appropriately sorting and releasing only sterile individuals of one sex, however, in many cases the harm is associated with both sexes. In these cases, an integrated pest management approach (i.e., removing fertile individuals from the target population and replacing them with sterile individuals) may be feasible as a means of augmenting the efficacy of a sterile-release approach without increasing the harm imposed by the released sterile individuals. However, IPM may not be feasible for many invasive species, especially those which are widely distributed over a large geographic area. The inefficiency of sterile-release is thus a barrier for its use to eradicate or control most invasive species.

Although the method of sterile-release may offer some utility for reducing or eradicating small populations of invasive species, more efficient genetic biocontrol methods are currently being explored that do not require the production of large numbers of individuals for release into the environment, and are not limited with respect to the size of the target population that can be targeted. Three of those approaches; YY Males, Trojan Female Technique, and gene drive; are considered in the following sections.

YY Males (Trojan Y Chromosome)

Hamilton (1967) is credited with proposing that an undesired population could be eliminated by shifting the sex ratio completely to a single sex. The idea that such an anthropomorphic sex ratio shift might be accomplished by aquaculture-induced sex reversal in fish first occurred to John Teem who hypothesized that sex reversal in a captive broodstock via use of exogenous sex hormones could be used to produce a genetically all-YY Male broodstock whose progeny could be released into an undesired population (Mills, 2009). An application of this concept, termed the Trojan Y Chromosome (TYC) approach was formally explored first in a mathematical

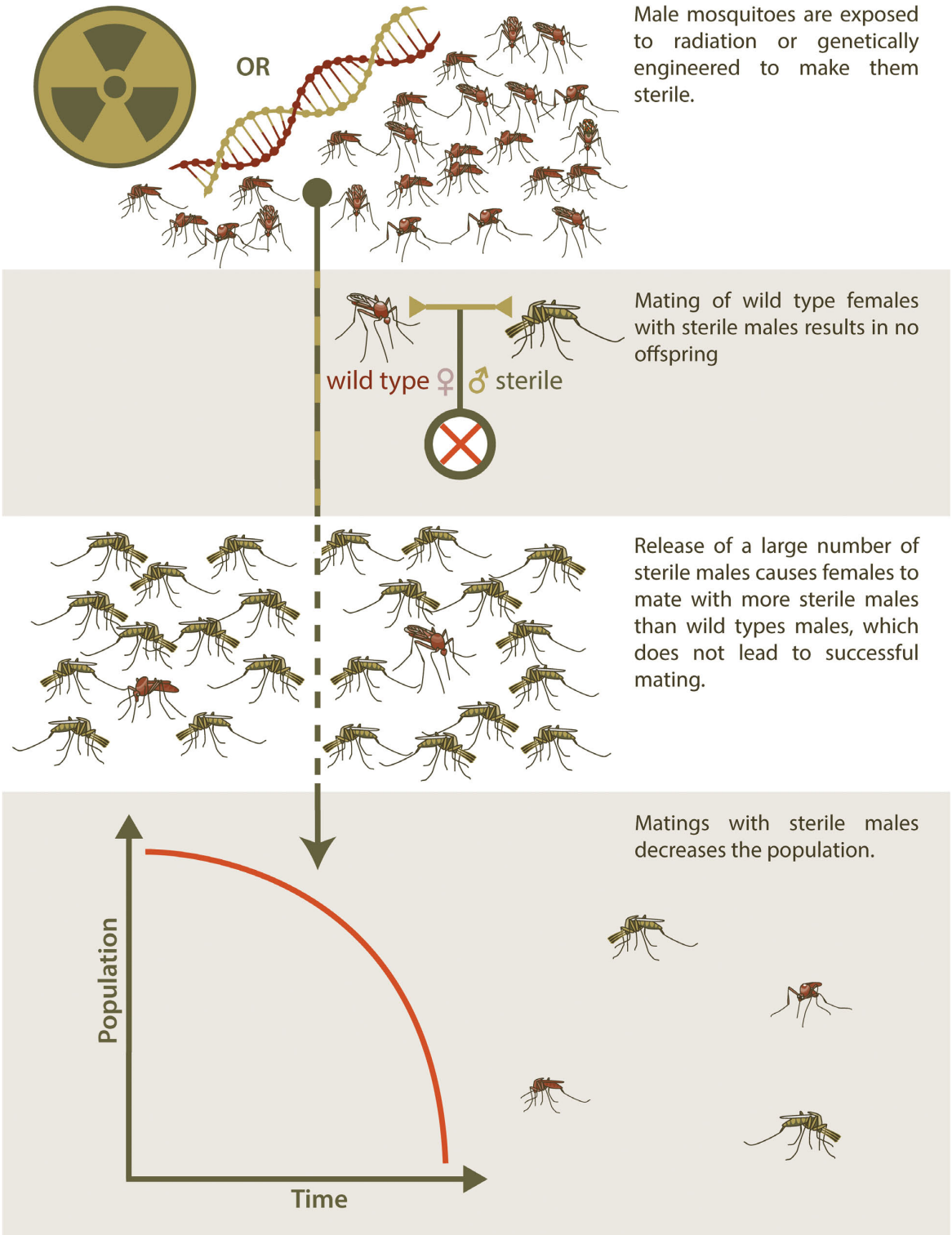

FIGURE 1 | Sterile Insect Technique. The release of sterilized males for population control is used to manage populations of invasive species through a method known as Sterile Insect Technique (SIT). Initially, a seed colony is maintained, from which batches of eggs are taken and amplified for several generations to produce large release cohorts. In traditional SIT, males are exposed to radiation to induce sterility, but newer technologies have made it possible to genetically engineer sterile males and potentially avoid the fitness and mating efficiency costs that accompany irradiation. Mating between these sterile males and wild type females do not result in offspring. These sterile males must be released into the wild population in very large numbers, so that wild type females are more likely to mate with sterile males than wild type males, in order to effectively reduce the population of the invasive species over time.

model evaluating the potential of the method for eradicating an invasive Nile Tilapia *Oreochromis niloticus* (L.) population (Gutierrez and Teem, 2006). In the TYC eradication approach, feminized, or egg-producing fish with two Y chromosomes are produced by commercial aquaculture practices involving selective breeding and sex-reversal by hormone treatment. These fish are then introduced into a target invasive fish population where they mate with normal males, giving rise to all male progeny, half of which will be sperm producing YY Males, further speeding the extirpation process (Teem and Gutiérrez, 2010). A variant of this original concept is to release sperm producing YY Males that would breed with wild females, resulting in all XY progeny (**Figure 2**). This approach is expected to be less efficient but also eradicated modeled invasive populations *in silico*, and was suggested to be more practical (Parshad, 2011), presumably because it would require the feminization of far fewer fish. Regardless of the type of YY Males being released, i.e., egg or sperm-producing males increase in the population over time at the expense of females until females are eventually eliminated, causing the population to collapse.

The development of a Trojan Y Chromosome broodstock for use in field studies as biocontrol agents was first undertaken for the Brook Trout (*Salvelinus fontinalis* (Mitchill), by the Idaho Department of Fish and Game (IDFG) in November 2008 (Schill et al., 2016). IDFG utilized the indirect broodstock development approach (Beardmore et al., 2001) and the use of a sex marker, PIT-tagging, and other production methods to develop a YY broodstock capable of producing large numbers of sperm-producing YY broodstock for field release in only three generations (Schill et al., 2016). In reporting their findings, the IDFG investigators preferred the use of the term "YY Males" over the previously used TYC label because it was more readily understood by the general public and decision-makers.

Having created the YY Male Brook Trout broodstock program in Idaho, population simulations were needed to provide guidance for field experiments and identify a range of likely stocking densities. The two most important predictions from the modeling exercises were the number of YY Male fish needed for release and the stocking duration in years to eradicate a target population (Gutierrez and Teem, 2006; Cotton and Wedekind, 2007; Stelkens and Wedekind, 2010). In addition, prior to 2016, all published TYC simulation authors (Gutierrez and Teem, 2006; Teem and Gutiérrez, 2010; Parshad, 2011; Parshad et al., 2013) had opted to evaluate only the addition of YY fish to invasive populations. None had evaluated other concurrent manual removal programs (hereafter suppression) as part of an integrated pest management program. Modeling of Brook Trout data from Idaho suggested that such a dual pronged IPM program would result in population extirpation within 2 to 4 years, assuming good YY Male fitness, and 5–15 years when YY Male fitness was only 20% that of wild males (Schill et al., 2017). Because stocking of YY Male fingerlings and manual suppression can readily be conducted at levels assumed in many of the simulations predicting complete eradication, Schill et al. (2017) recommend full-scale field testing of YY Male stocking in both streams and lakes within an IPM program that includes manual suppression.

Concurrent with the modeling exercises, Kennedy et al. (2017) conducted a pilot study to determine if stocked YY Male Brook Trout can survive, emulate the spawn timing of wild fish, reproduce with wild fish, and produce only XY males. YY Male Brook Trout were evenly dispersed in each of four pilot study streams in a single year and comprised an average of 3.1% of adult Brook Trout at spawning time several months later. Subsequent genetic assignment testing of Age 0 Brook Trout fry demonstrated that an average of 3.7% of fry collected the

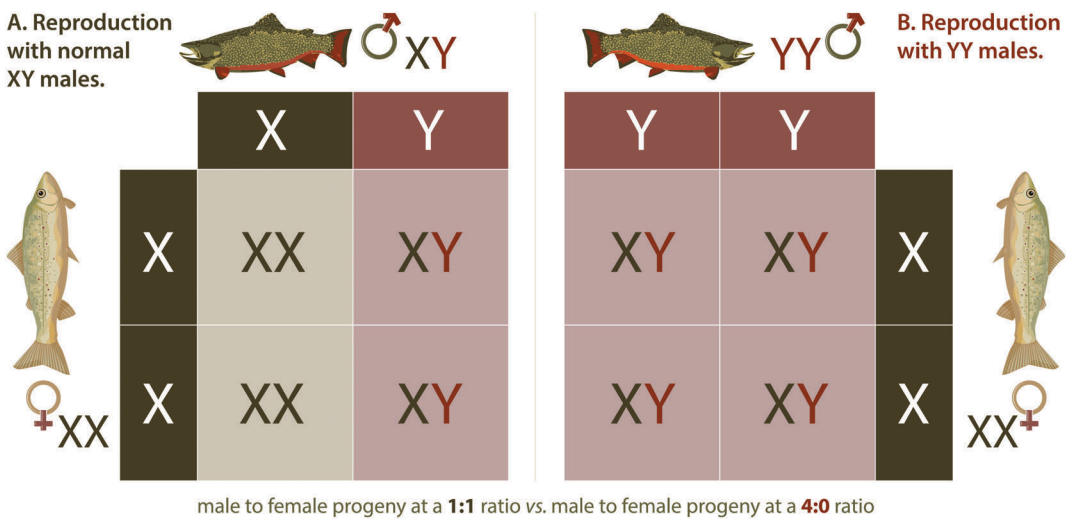

FIGURE 2 | YY Males. Inheritance of sex chromosomes in Brook Trout **(A)** follows typical Mendelian patterns. Mating between a wildtype male (XY) and wild type female (XX) results in a 1:1 ratio of female (XX) to male (XY) fish in the offspring. When a YY male Brook Trout mates with a wild type XX female **(B)**, the only potential resulting genotype is male (XY), meaning all offspring are males. Continued introduction of YY Males in adequate numbers theoretically results in eradication of invasive Brook Trout populations over time.

following summer were the progeny of YY Males and all were XY males, confirming that stocked YY Male fish can survive and spawn successfully with wild females and produce all-male progeny (Kennedy et al., 2018). Based on the positive pilot study results, IDFG subsequently expanded YY Male research efforts to full-scale field evaluations involving 13 waters including six alpine lakes and seven streams. The initial results of this research effort are just beginning to be documented.

The YY Male eradication technique offers an approach to eradicate invasive fish that does not use genetic engineering and is currently the only genetic biocontrol utilized in the United States. This technique is currently supported by fisheries resources managers in several western United States. If successful, it may provide a model for the development of other types of genetic biocontrol that similarly avoid the use of transgenics and are potentially applicable to a large number of invasive species.

Trojan Female Technique

The Trojan Female Technique, or TFT, is a novel twist on the sterile insect or sterile male approach. In the TFT, sustained population control is achieved through the steady release of "Trojan females" that carry mitochondrial DNA (mtDNA) mutations that cause reductions to male, but not female fertility (Gemmell et al., 2013; **Figure 3A**).

The TFT concept is enabled because of an evolutionary loophole that is common to most eukaryotic life. mtDNA is overwhelmingly inherited maternally thus mtDNA mutations that affect only males will not be subject to natural selection (Frank and Hurst, 1996). Theory predicts that such mutations can reach high frequencies in natural populations potentially impacting on their viability (Gemmell and Allendorf, 2001); an idea termed "Mother's Curse" (Gemmell et al., 2004). If individuals carrying such naturally occurring mutations could be identified and cultivated, then the release of females carrying these mutations could, at least in theory, achieve self-perpetuating population control (Gemmell et al., 2013).

A variety of naturally occurring mtDNA mutations that reduce male, but not female, fertility have now been identified in fruit fly (Xu et al., 2008; Clancy et al., 2011; Yee et al., 2013; Patel et al., 2016), mouse (Trifunovic et al., 2004; Nakada et al., 2006), and European hare (Smith et al., 2010). The existence of these mutations in other species has not yet been extensively investigated. Given the ubiquity and conservation of mtDNA, it seems likely that these mutations occur in other species.

Modeling studies suggest that the TFT has the potential to achieve pest control under a wide range of conditions (Gemmell et al., 2013). Single large releases (10% of the population) and relatively few small repeat releases (1% of the population) of Trojan females both provided effective and persistent control within relatively few generations (**Figure 3B**). Although greatest efficacy was predicted for high-turnover species, the additive nature of multiple releases made the TFT applicable to the full range of life histories modeled. TFT mutations became increasingly less effective when males carrying Trojan mtDNA were only partially infertile, having lower fitness to wildtype males. Multiple female matings also reduced the effectiveness of the TFT (Gemmell et al., 2013).

Recent work in fruit flies (*Drosophila melanogaster*) supports the view that the TFT can reduce populations (Wolff et al., 2016). However, the level of population suppression achieved was modest, 8% across 10 generations, thus the TFT would need to have a stronger effect to have utility in the field. The search for TFT mutations that have stronger effects continues but is limited by the standing genetic variation in the populations that are being screened and remains time consuming. Thus, while one of the original strengths of the TFT approach is that it uses naturally occurring mtDNA variants (Gemmell et al., 2013) and, thus does not involve genetic engineering, directly or indirectly engineering one or multiple mutations into the mtDNA may enable more rapid discovery of TFT mutations.

Directly engineering mutations into mtDNA is far from trivial (Gammage et al., 2018a). Some researchers have reported success in modifying a series of nucleases to work efficiently in the mitochondria to cut and thus eliminate a defective mtDNA copy (Gammage et al., 2018b). However, the general inability to import nucleic acids into mitochondria severely limits the prospect of more direct manipulation using CRISPR based gene editing to introduce novel genetic variants – particularly beyond lower metazoans (Gammage et al., 2018b).

An alternative approach is to generate lines of animals that have defects in the polymerase responsible for the replication of mtDNA to rapidly develop and explore new mtDNA variants. Mice genetically engineered to express a proof-reading-deficient version of PolgA, the nucleus-encoded catalytic subunit of mtDNA polymerase, show a heightened incidence of *de novo* mtDNA mutation (Trifunovic et al., 2004). These mice have successfully been used to investigate the role of mtDNA in longevity (Vermulst et al., 2008) and disease, and may be a means through which novel mtDNA mutations can be generated and subsequently explored for male specific effects on fitness. Recently, 12 new mouse mitolines were established using PolgA founders and are currently assessing the effects of the mtDNA mutations we generated on fertility (Gemmell et al. unpublished). A similar experiment is now underway in fruit flies (Kauppila et al., 2018). However, this process relies on random mutation which is much less efficient than targeted gene editing approaches.

A potential middle ground approach is to directly identify mitochondria carrying desired mutations *in vitro,* and then transfer these mitochondria directly into developing zygotes (Nakada et al., 2006) or capture these using backcrossing experiments (Yu et al., 2009; Tourmente et al., 2017). Through such an approach, Nakada et al. (2006) developed a transmitochondrial mouse model (mito-mice) that carried wild-type mtDNA and a mutant mtDNA with a pathogenic 4,696 bp deletion (ΔmtDNA). Refinements on this approach, wherein mtDNA variants are generated using classic molecular biology or recent synthetic biology approaches, could establish a framework to target specific sites in the mtDNA and test their effects on male and female fertility, and ultimately their potential application in the TFT.

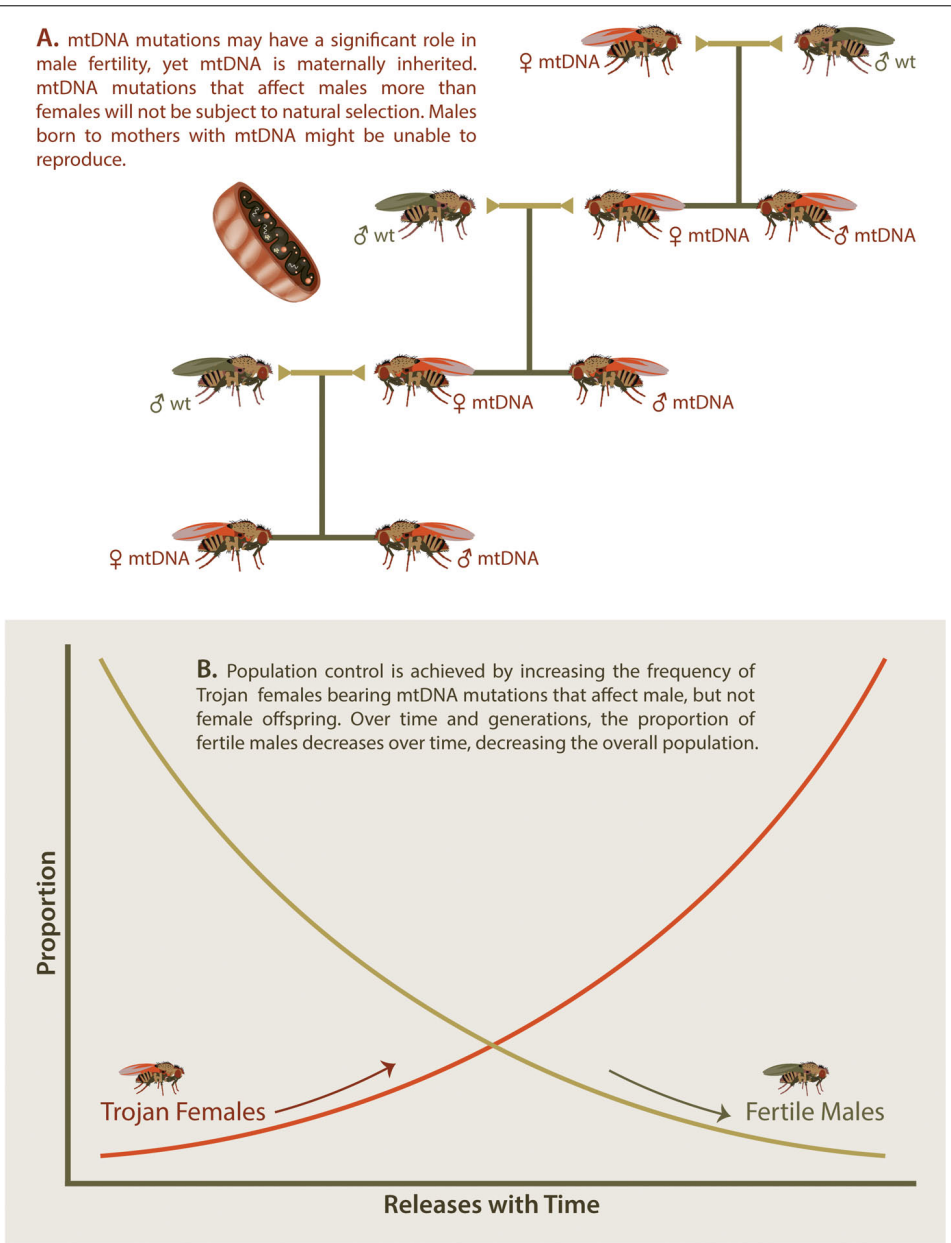

FIGURE 3 | Trojan Y Females. Several mitochondrial DNA (mtDNA) mutations have now been identified that cause significantly reduced male fertility while having no effect on female fertility **(A)**. If females carrying such mtDNA mutations are introduced into wt populations, then male offspring will demonstrate reduced fertility while female offspring will continue to pass on the mtDNA to future generations. **(B)** Introduction of Trojan females increases the portion of Trojan females over time and leads to a concurrent decrease in fertile males over time. This decrease in fertile males will cause an overall decrease in the targeted invasive species in a similar manner to that observed with sterile insects.

Although the TFT shows promise as a species-specific, reversible, and humane form of population control that has more support from the public than many alternative technologies (Gemmell et al., 2013), there are substantial hurdles to be overcome. Foremost among these is that the effects observed via empirical experimentation are weak, such as only 8% fruit fly population reduction across 10 generations (Wolff et al., 2016) and has yet only modest effects observed on mtDNA type on mouse fertility (Tourmente et al., 2017).

COMPROMISING EFFICIENCY TO GAIN CONTROL

These methods, YY Males and TFT, are likely more efficient than sterile release, requiring substantially fewer Trojan individuals to be introduced into the environment in order to effect a change on the target population. Each strategy also provides natural resource managers with some measure of predictability and control in the eradication process, a feature that may be

lacking in other approaches (e.g., gene drives). Both strategies require that a steady influx of Trojan individuals are added to a target population to cause eradication over time. However, by ceasing the addition of Trojan individuals, eradication efforts can be terminated. Having a means of terminating an eradication program is a feature that is important to natural resource managers as it reduces the risk of making mistakes that permanently change the population. Neither method involves the introduction of transgenic organisms or GMO's into the environment (Cotton and Wedekind, 2009; McNair et al., 2015), which will likely be viewed as advantageous by resource managers. In contrast to TFT, the YY Male technology has been developed to the point of practical application and field testing for Brook Trout is ongoing in Idaho and three other western United States states including Oregon, Washington, and New Mexico. The TFT genetic biocontrol has not yet been developed sufficiently to allow practical application against invasive species. In theory, it should be broadly applicable to a variety of invasive species provided that the mitochondrial genome in the organism can be engineered. Unfortunately, genetic engineering of mitochondrial genomes is currently impractical, so the future benefit of the TFT strategy for invasive species genetic biocontrol has yet to be realized. More research is needed in mitochondrial genome engineering to determine if this non-transgenic approach can be applied more broadly to any invasive species other than fruit flies.

Gene Drive

Gene drives are genetic elements with biased inheritance and have considerable potential for suppression of target pest populations (Burt, 2003; Sinkins and Gould, 2006). While naturally-occurring gene drives have been identified (e.g., T allele in *Mus musculus* L.), the recent advent of CRISPR/Cas9 gene editing technology has enabled generation of synthetic gene drives that in theory could be adapted for use in any sexually reproducing species (Esvelt et al., 2014). To date, most synthetic gene drive development has been performed in insect species including the experimental model *Drosophila melanogaster* Meigen (Gantz and Bier, 2015) and the malarial vectors *Anopheles stephensi* Liston (Gantz et al., 2015) and *Anopheles gambiae* Giles (Hammond et al., 2016, 2017). The relative success of these studies has generated considerable excitement in the conservation technology community and gene drives have been proposed as a "silver bullet" for eradication of invasive mammalian pests. However, despite their potential, efficient CRISPR gene drives have yet to be developed any vertebrate species outside of cage experiments.

At the molecular level, a synthetic gene drive consists of an expression cassette encoding a site-specific endonuclease (e.g., the CRISPR/Cas9 system). Importantly, this cassette is inserted into a chromosome at the genomic site that is cut by the endonuclease. Once the cassette has been integrated, the chromosome becomes immune to cleavage. Thus, a cell that is heterozygous for a gene drive cassette contains one allele that is susceptible to digestion by the endonuclease [the wild type (WT) allele] and one allele that is not (the gene drive allele). Expression of the gene drive endonuclease in a heterozygous cell will generate a double stranded break in the WT allele (**Figure 4A**). Repair of the double stranded break by homologous recombination (using the gene drive allele as a repair template) results in conversion of the WT allele to a gene drive allele, in a process termed "homing," which renders the cell homozygous for the gene drive allele. Homing can be restricted to the gamete (egg/sperm) precursors resulting in selective homozygosity in the germline (i.e., the somatic cells remain heterozygous). Alternatively, homing can be directed to occur in the zygote (one-cell embryo). The homing event will ensure that the gene drive allele will be present in all gametes and will be passed on to all progeny. Over several generations, gene drives can spread rapidly through a given population (**Figure 4B**). While maximum gene drive spread occurs with 100% transmission, any increase above Mendelian (50%) transmission can still promote gene drive propagation throughout the entire population. Remarkably, gene drive transmission in mosquitos can be as high as 99.7%, indicating that CRISPR-mediated homing can be very efficient in insects (Gantz and Bier, 2015; Gantz et al., 2015; Hammond et al., 2016). However, gene drives with potential for field deployment are yet to be developed in rodents.

Other novel gene drive strategies based on innovative applications of CRISPR/Cas9 genome editing include the Y-CHOPE (Y-Chromosome deletion using Orthogonal Programmable Endonucleases) strategy (Prowse et al., 2019). This approach utilizes a standard homing cassette that also incorporates a programmable endonuclease that "shreds" the Y chromosome, thereby converting XY males into fertile XO females. The "shredding" of the Y chromosome using Cas9- or Cas12a-gRNA complexes that target repeat sequences on the Y chromosome has been demonstrated in embryonic stem cells (Adikusuma et al., 2017; Prowse et al., 2019). *In silico* modeling demonstrated that a Y-CHOPE gene drive can eradicate a pest vertebrate population. However, simulations indicate that, for polygynous species such as mice, Y-shredding efficiencies must be greater than ∼90% to produce high probabilities of eradication success (Prowse et al., 2019). Y-CHOPE may provide a useful alternative to the homozygotic XX sterility and homozygotic embryonic non-viability drives described above.

In silico modeling indicates that gene drives targeting female fertility genes and embryonic viability genes may be useful strategies for invasive mouse population suppression, and that a novel Y-shedding gene drive strategy has eradication potential (Prowse et al., 2019). Before any of these approaches can be considered for deployment, extensive engagement with stakeholders, regulators and the general community is essential. In addition, proof-of-concept studies in laboratory mice are required for development of field-ready tools. The time and effort required for technology development in mice should not be underestimated. It will likely be several years before the true potential of CRISPR gene drive technology in rodent can be fully appreciated. To date only a single paper has been published on gene drive homing in mice (Grunwald et al., 2019), and considerable optimization is required before this technology can seriously be considered for trials testing the ability to control mice populations. Non-homing approaches for rodent management such as X-shredder (McFarlane et al., 2018) and Cleave and

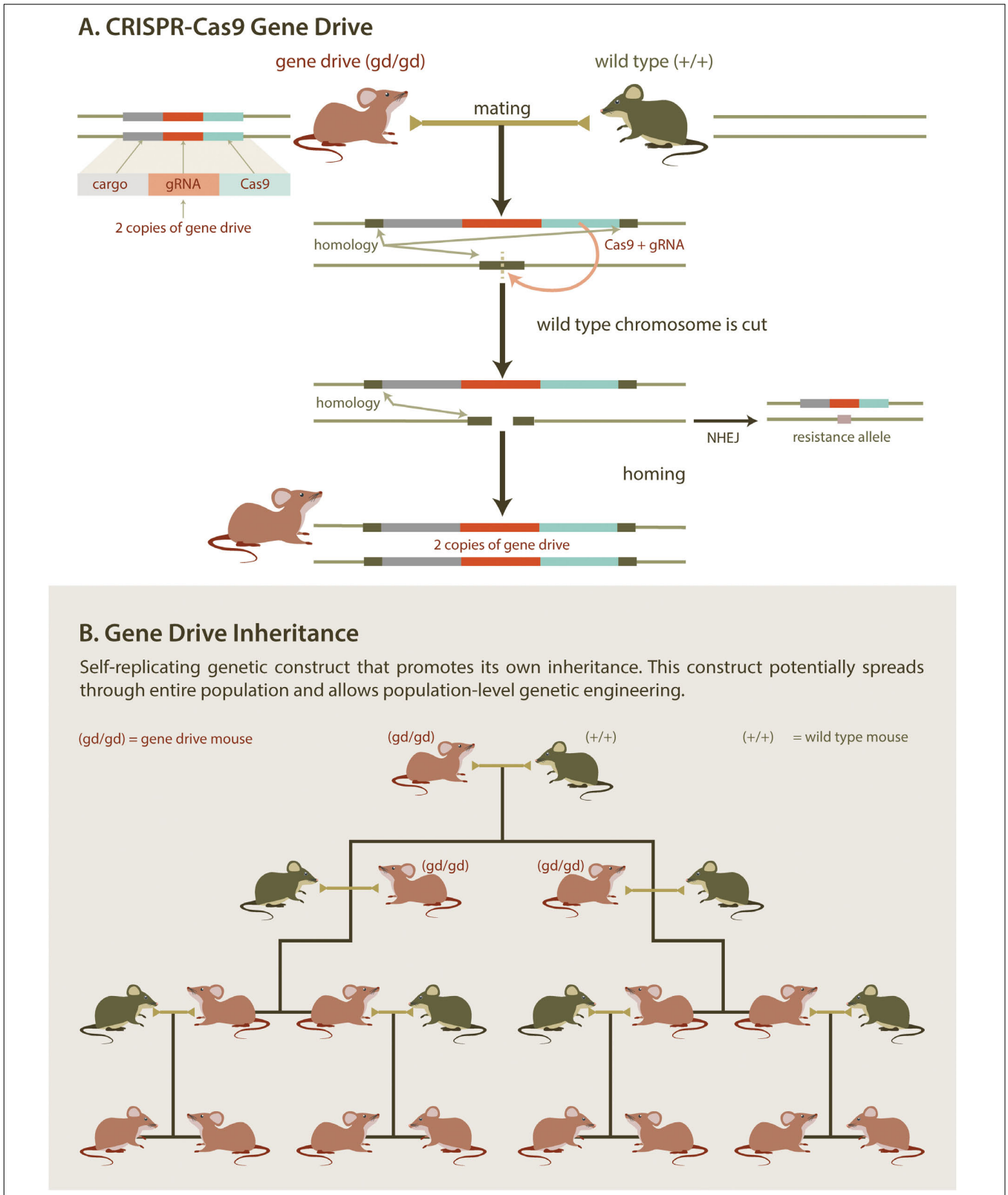

FIGURE 4 | Gene Drive. CRISPR/Cas gene editing technology provides a practical new method to introduce genetic elements that bias inheritance. Mating mice containing a self-replicating gene drive element with wild type mice **(A)** results in offspring with two copies of the gene drive element. If the drive mechanism is efficient **(B)**, this allows for a desirable trait to be spread through an entire population. Desirable traits could include genes designed to reduce populations by, for example, skewing sex ratios.

Rescue (Oberhofer et al., 2018) strategies are therefore also worth exploring for conservation objectives.

Containment of Gene Drives

Testing of gene drives on islands

Gene drives have been proposed as powerful tools for controlling pest populations yet remain controversial. Perhaps the greatest unique risk potentially associated with these technologies is spread beyond the pest population which is being targeted (termed "transgene escape"), possibly affecting non-target populations or species. For species which are not "global targets" (i.e., those where the entire global population is the target), appropriate measures should thus be taken to reduce this risk, if these technologies are to be trialed in the field (Harvey-Samuel et al., 2019).

Transgene escape can occur in one of two ways. The first of these occurs at the spatial level in which the gene drive could move into a non-target population of the target species, for example from an area where the species is an invasive pest, back into the native range of that species. This is termed "intra-specific transgene escape" (Harvey-Samuel et al., 2017). Secondly, the gene drive could move into a closely related species at the release site (inter-specific transgene escape). These characteristics are most easily satisfied by oceanic islands, but sufficiently isolated ecological islands may also be sufficient.

Intra-specific transgene escape

Regardless of their mechanism, all gene drive systems are vertically transmitted technologies requiring gene flow to spread. Therefore, the level of gene flow between target and non-target populations a critical parameter in determining whether a gene drive transgene will escape a particular target population. Intra-specific transgene escape must therefore involve migration of individuals from the release area to non-target populations and subsequent introgression of their genomes into those populations. If sufficiently isolated from non-target populations, islands (geographical isolation or genetic isolation) provide high levels of ecological gene drive containment. The degree of containment offered will generally grow with increasing geographic distance between two populations, increasing ecological inhospitality of the intervening area and decreasing size of the population at the release site. In summary, small target populations surrounded by large distances of inhospitable terrain (including ocean if the target is non-marine) are the least likely to escape. Of course, what is deemed to be a sufficiently small population, or a great enough distance will be highly dependent on the ecological characteristics of the target species such as its reproductive rate and dispersal abilities. Additionally, possible human-mediated dispersal must be considered and should be minimized by careful trial site selection, biocontainment, and biosecurity.

An empirical field example of ecological containment can be found in the trialing of *Wolbachia*-based gene drives spreading through *Aedes aegypti* mosquito populations in Australia (Hoffmann et al., 2011; O'Connor et al., 2012; Hoffmann and Broadhurst, 2016; Schmidt et al., 2017) where release sites of *Wolbachia* infected mosquitos were separated from ecologically hospitable areas by a relatively impassable barrier. The gene flow between the two populations was sufficiently restricted such that the drive would not spread effectively between *Wolbachia* infected and native populations. However, when release sites occurred within a larger contiguous population, gene flow occurred at a high enough rate for the gene drive to spread beyond the target site and invade the wider population.

Another characteristic of island populations that reduced inter-specific escape is their relatively low genetic diversity and, specifically, the high frequencies of fixed alleles arising from the founder effect and subsequent drift in small initial generations. This can be advantageous for licensing sequence-specific drives (e.g., those based on CRISPR technology) to that particular target population, if a sequence can be identified which is fixed in the target population but not in non-target populations. This "genetic gene drives containment" remains robust even if the targetable sequence is present in non-target populations (Sudweeks et al., 2019).

Inter-specific transgene escape

Another potential route for transgene escape is through hybridization between the target species and a closely related species at the release site, followed by introgression of gene drive alleles by hybrids back into the non-target species. As with intra-specific transgene escape, there are several factors which can aid in reducing inter-specific transgene escape at island site locations. First, for hybridization to occur, there must be a closely related species (most likely a congenic species) at the release site. The risk of inter-specific transgene escape can thus be drastically reduced by choosing locations which are devoid of species congenic with the target. In some cases, the nature of hybridization events and the mechanics of the drive may also preclude transgenic hybrids being formed, even if congeners are present. For example, if hybridization is unidirectional with regards to sex and does not involve target species males, then drive systems which convert genotypic females to phenotypic males (such as Y-drive) (Burt, 2003) will be limited to the target population.

Secondly, there are factors which can act cumulatively to limit or prevent the risk of a drive spreading even if it does enter a non-target species. For example, if hybridization events do occur, they must result in fertile and competitive individuals that are able to introgress the drive into the non-target species. If hybrids are competitive, but hybridization events are rare, it is possible that stochastic loss of the drive will occur prior to the hybrid individuals being able to introgress the transgene into the non-target species. Similarly, for drive designs which require a minimum population frequency in order to spread, (Marshall and Akbari, 2018) the rate of this introgression may fall below that necessary for invasion of the non-target species. Finally, if competitive hybrids can introgress gene drive alleles into a non-target species at a relatively high rate, the drive would need to remain functional in this foreign genome. Even if regulatory components utilized to build the drive were compatible with the foreign species, loci targeted by the drive would also need to be present. The degree to which even small changes at the target locus can impair the spread of drives through relatively homogenous lab populations suggests that this scenario will be

relatively unlikely. The simple nature of island communities means that examples where no closely related species occur will be relatively frequent, and that surveys of these communities to assess the risk of inter-specific transgene escape will be relatively easy to complete.

Gene drive system development is underway in several pest vertebrates. If promising technologies are to be trialed in the open field, there are advantages to conducting these trials on islands – whether oceanic or purely ecological. Small, ecologically simple, geographically isolated and genetically distinct island locations can aid in reducing the risk of unintended movement of the drive system into a non-target population. Islands offer the additional advantage of potential genetic containment through the exploitation of locally-fixed alleles.

Genetic Containment of Gene Drives – Locally Fixed Alleles

In order to reduce the uncertainties associated with the spread of gene drives, modifications to gene drives have been suggested that eliminate their capacity to propagate in a self-sustaining fashion, and instead allow them to persist for only a limited duration or in a limited area such as suggested by the LFA approach (Sudweeks et al., 2019). Daisy-chain gene drives (Noble et al., 2019) have been suggested as methods to limit the duration and spread of a gene drive. These techniques would have particular relevance to gene drives targeting invasive species because they would further mitigate the risk of unintended spread of the gene drive back to the native population.

Invasive mice on islands, which cause ecological destruction to sensitive island ecosystems (Campbell et al., 2019), are a good model system for exploring genomic targets for gene drive that could be specific to that population. Classic population genetic theory of founder events, genetic drift, and allelic fixation are the basis of assuming that invasive mice on islands, would have "locally-fixed alleles" that could then be used as targets for gene drive gene editing. A newly founded population of commensal rodents that invade an island from a ship would eventually lead to an island population that would have different allele frequencies than found in the continental population from which they originated. Further, some alleles might be fixed, found in each member of the population, due to genetic drift. These may not be alleles that are not found in the original populations, but they would occur in a much higher frequency (up to 100%) than the source populations. Identifying and exploiting such "locally-fixed alleles" would make a gene drive effective specifically in that island population and thus not be a risk for accidental spread to other geographical regions.

Mathematical models can be used to assess the effectiveness of a gene drive associated with an island population of locally-fixed alleles. Further, they can be used to test the spread of a drive in a continental population where the islands locally-fixed alleles may occur in low frequency. For gene drives to be effective, the target allele must be fixed in the island population meaning they are found at 100% frequency on the island. It is important to test this event as the possibility exists of a gene drive mouse getting transported to the continent as humans have long managed to transport commensal rodents unintentionally. Mathematical models demonstrate that, for a gene drive associated with an island locally-fixed allele, escape of a drive-bearing individual to from an island that was introduced into a continental population with that allele in low frequency would cause only a temporary decline in the continental population with a subsequent rebound in population numbers (Sudweeks et al., 2019). This suggests that if locally-fixed alleles are identified in an invasive, mouse island population that they could indeed serve as a biologically limiting gene drive target. It should be noted that mathematical models by Noble et al. (2019) indicated that gene drive systems have a highly likelihood for invasiveness into wild population, so contained field trials could have unintended spread of gene drive to other populations.

To investigate the feasibility of the locally-fixed alleles strategy, authors of this paper KO and AP used previously published genomic data to characterize the frequency of such alleles in an invasive island mouse population and a putative continental source population. As a part of their survey of wild *Mus* populations, Harr et al. (2016) compiled a whole-genome resequencing dataset that included samples (N = 3) of *M. musculus helgolandicus*, a subspecies found only on the German island of Heligoland, where it evolved from anthropogenic introductions of *M. m. domesticus* (Babiker and Tautz, 2015). We contrasted genomic variation in these samples with *M. m. domesticus* (N = 8) collected near Cologne-Bonn, Germany (Pezer et al., 2015) to identify locally-fixed alleles and characterize population differentiation. After downloading the aligned sequence files[1] we first estimated genome-wide diversity as expected single-nucleotide polymorphism heterozygosity (SNP-H_e) (Fischer et al., 2017) based on over 135 million autosomal sites. Consistent with the expectations for an isolated island population subject to a population bottleneck, *M. m. helgolandicus* mice had substantially lower levels of allelic diversity (SNP-H_e = 0.078) compared to continental mice (SNP-H_e = 0.315) in this new work. To assess population genetic differentiation, we calculated the fixation index (F_{ST}) using the *poolfstat* R package (Hivert et al., 2018). Estimates of genome-wide allelic variation confirmed substantial population genetic differentiation (mean F_{ST} = 0.136) despite a relative short time (ca. 400 years) since island colonization and likely in the presence of persistent gene flow (Babiker and Tautz, 2015), which broadly points at the role of founder effects in population divergence (Carson and Templeton, 1984). More importantly to the present discussion, these results suggest that island colonization can have genetic effects on rodent populations that may result in viable genomic targets for population-specific synthetic gene drives. In this case, we scanned the genomic datasets for polymorphisms that created functional Cas9 protospacer adjacenet motif (PAM) sites in island populations but were absent in continental mice. Previous work has suggested that a single mutation within the PAM site is sufficient to preclude Cas9 genome editing activity (Hsu et al., 2013). Our analysis found 6,499 functional Cas9 PAM sites in Heligoland mice that were at absent in contential mice. Of these, 2,915 occurred in intergenic regions, which is desireable due to

[1] http://wwwuser.gwdg.de/~evolbio/evolgen/wildmouse/

the reduced likelihood of unanticipated phenotypic effects in mice bearing the gene drive cargo (Prowse et al., 2017). Thus, the results of this pilot study suggest that genome engineers may have ample targets around which a gene drive could be designed for population-specific use in island populations, though we note the preliminary nature of these findings due to the low sample size. Selection of optimal target(s) from this pool of candidates should maximize efficacy while minimizing risk. Existing bioinformatic toolkits (e.g., VARSCOT)[2], leverage machine learning and genetic/epigenetic diversity to compare efficacy amongst targets and to assess the likelihood of off-target and non-target effects (Cameron et al., 2017).

The locally-fixed allele strategy depends critically on identifying sites that are completely fixed in the target population, as rare variants in the PAM site will essentially create resistance alleles to the gene drive. Future efforts to screen genomic variation in island populations should carefully consider sampling strategies that will afford the greatest confidence in allele frequency estimates. Notably, evidence suggests that experimental designs that pool population samples prior to sequencing (i.e., "pool-seq") may provide greater precision in allele frequency estimates compared to deep sequencing of a small number of individuals (Rode et al., 2018). To enable this, an expanded statistical analysis pipeline for pool-seq data has been developed (PeSTo)[3], improving the power and speed of the original pool-seq pipeline (Anand et al., 2016). With access to sufficient population genomic data (within and between populations, target and related non-target species), an analytical pipeline that both identifies and evaluates targets for efficacy and risk can be linked to models of in-field propagation to conduct "digital" risk assessments of genetic control technologies that have yet to be developed by genome engineers.

RISK ASSESSMENT

Risk Assessment for Classical Biocontrol

Both genetic biocontrol and classical biocontrol involve the introduction of a new organism into the environment. Classical biocontrol is the release of a natural enemy to manage an introduced invasive species. Classical biocontrol agents are released with the understanding that the newly introduced organism will become permanently established in the environment. Because biocontrol effects of an introduced biocontrol organism are permanent and cannot be reversed, risk assessments for classical biocontrol agents must carefully consider risks that the new organism may pose to the environment. It is thus worthwhile to examine the risk assessment strategies used for classical biocontrol organisms and consider this risk assessment framework as a possible model for genetic biocontrol organisms.

Historically, classical biocontrol has been successfully applied to manage invasive species all over the world (Messing and Wright, 2006). Host specificity is the primary concern when considering the introduction of a biocontrol agent (McEvoy, 1996). Modern biocontrol programs require extensive host specificity testing in a quarantine environment and non-native organisms cannot be released without oversight from a rigorous scientific and regulatory vetting system (USDA, 2017). In the United States, this petition for release is submitted to the United States Department of Agriculture, Animal and Plant Health Inspection Service (USDA-APHIS) Biocontrol Program, with oversight from the Technical Advisory Group for Biological Control of Weeds (TAG-BCAW), which includes Canada, Agriculture and Agri-Food Canada, Mexico SAGARPA-SENASIA-DGSV, and other United States governmental agencies as cooperating organizations (USDA, 2017), allowing collaboration across North America. This strategy of testing and oversight has led to hundreds of successful biocontrol releases (Coulson, 1992; Hajek et al., 2007; Cock et al., 2016). To harmonize the regulation of invertebrate biocontrol agents in Europe, the International Organization for Biological Control of Noxious Animals and Plants (IOBC) organized the national regulatory framework on invertebrate biocontrol agents from across Europe into one guidance document (Bigler et al., 2010). Additionally, the FAO IPPC has established guidelines for the release of biocontrol agents (FAO, 2017).

When successfully implemented, classical biocontrol is an environmentally safe and cost-effective alternative to chemical pest control (van Lenteren et al., 1997; Bale et al., 2008). New technologies are becoming available to increase the effectiveness of classical biocontrol, such as sterile insect technique and gene drives. These new modified biocontrol strategies are the next step in the very successful evolution of biocontrol over the last century. The risk assessment framework for the release of classical biocontrol agents has a proven track record and offer a model for the release of many genetic biocontrol agents.

Risk Assessment for Genetic Biocontrol

In order to establish whether an organism created using genetic biocontrol would pose a risk to the environment, it is necessary to perform an environmental risk assessment, a systematic procedure for predicting the possible harm that a hazard may pose to human health or the environment. A well-established framework exists for assessing the risk associated with the introduction of genetically modified plants into the environment (Raybould, 2006, 2007; Andersson et al., 2010; Garcia-Alonso and Raybould, 2014) as well as for classical biocontrol discussed in the previous section. These same frameworks can also be used to assess the potential risks associated with the introduction of a genetic biocontrol organism. Any of the approaches are not a one-size-fits all solution and will need to be evaluated for the specific situation in which they are used.

For release of genetic biocontrol agents, the first step in risk assessment will be problem formulation, where entities of value within the environment (also known as protection goals) that could be harmed by a gene drive are identified. Potential pathways leading to harm of the protection goals by the gene drive are then hypothesized (resulting in a risk hypothesis) and experimental data is collected to either validate or disprove the risk hypothesis. If experimental data suggests that no harm is

[2]https://github.com/BauerLab/VARSCOT

[3]https://bitbucket.org/toolsforpools/pesto/

likely to occur, this would provide information to decision-makers that the environmental risk posed by the new organism is low. Alternatively, the experimental data could indicate that the risk of harm is high, leading decision-makers to prohibit release of the new organism (or seek mitigation measures to reduce the risk to an acceptable level).

When considering release of genetic biocontrol agents, the first risk that needs to be assessed is whether release of a large population of reared individuals will have an impact on the environment, a risk assessment process similar to that done with classical biocontrol agents. This risk assessment can be done following the current framework for release of classical biocontrol agents. Release of mass-reared organisms is well established and conducted as part of area-wide IPM programs, and there is a history utilizing genetic biocontrol, such as SIT and *Wolbachia* infected insects, as part of such strategies (Mumford, 2012). When the organism is genetically modified, the environmental risk assessment should be done on a case-by-case basis. However, the risk assessment framework used for the release of mass reared insects and invertebrates helps to ensure that the appropriate science-based evidence is provided for the environmental risk assessment.

Gene drives may present a challenge for risk assessment in several ways. First, it will be necessary to clearly define what constitutes the "harm" that a gene drive poses to a protection goal in the environment. Spread of a trait within the environment may not itself constitute actual harm, but instead simply represents an event. Risk assessors will thus need to make a clear distinction between what constitutes harm and what does not. Another challenge for risk assessment of gene drives will involve testing risk hypotheses with experimental data. Trialing of gene drives will require a very high level of containment (ideally both physical containment and genetic containment). Although physical and genetic containment of gene drive mice on islands seems feasible, it may be difficult to find appropriate test conditions for gene drives constructed in other species. Mathematical modeling may therefore be an important means of providing data to inform risk assessments for gene drives for some invasive species. It should be noted that the regulatory framework noted above for classical biocontrol may not apply to genetic biocontrol and management of invasive fish and wildlife. In particular, in the United States, with the exception of migratory waterfowl and ESA listed species, wildlife (whether native or invasive) in the United States is owned, protected and managed by each of the states (Smith, 2011). This risk assessment framework that USDA-APHIS uses for classical biocontrol might not be relevant when considering genetic biocontrol for management of invasive vertebrate wildlife species in the United States.

PUBLIC PERCEPTION OF GENETIC BIOCONTROL

Although scientific and regulatory hurdles exist for the practical use of genetic biocontrol to control invasive species, perhaps the greatest hurdle to be overcome will be public acceptance of the technology. Gaining public trust will also be an essential component in the development of new genetic biocontrol methods (and was identified in the workshop as the major barrier to implementation any genetic biocontrol).

The prospects for the development of genetic biocontrol to control invasive species will likely hinge on public perception of whether the use of such new technologies is sufficiently warranted to solve the problems being addressed. In a recent review of genetic control methods for invasive species, YY Male (TYC) was identified as the method least likely to generate public controversy (Thresher et al., 2014) though TFT and gene drives were not considered at that time. However, a recent Pew Research Center study (Funk and Hefferon, 2018) indicates public attitudes toward the use of genetic engineering on animals tend to be supportive if the technology is being applied to a major human health issue (e.g., preventing disease transmitted by mosquitoes). The public was less supportive of other uses involving the environment (e.g., increasing meat production for agriculture or recovering extinct species as a means of restoring biodiversity).

Whether the general public would consider the eradication of invasive species a problem that warrants the use of genetic engineering is yet undetermined. The review by Oliva et al. (2014) provides a historical context for how public perception has negatively impacted the implementation of classical SIT for vector management. A study on public perception of genetic biocontrol to control invasive fish found that a majority of people in the Great Lakes region were in favor of using genetic biocontrol to manage invasive fish populations, but recommended regulatory systems for the industry to help mitigate unintended consequences (Sharpe, 2014). In Mali, the public was open to the release of genetically modified mosquitoes to manage malaria, but wanted assurance that there would be no negative environmental or human health consequences (Marshall et al., 2010).

A landscape analysis on the use of gene drives in mice showed that the research community was concerned that gene drives would only receive public support if they could effectively eradicate the species, with a general concern that if implementation of gene drive were only partially successful, the public support for the strategy and even research on gene drive would be greatly diminished (Farooque et al., 2019). In a larger study of the public, the majority of the respondents were against gene editing of wildlife (71.3%), while 38.5% thought that using gene editing to control invasive species was not morally acceptable (Brossard et al., 2018). In general, there appears to be more support for genetic biocontrol of human disease vectors, but less support for management of wildlife. However, little was known about the perceptions of gene drive in agricultural systems. In a survey of over 1000 members of the public, Jones et al. (2019) the majority of respondent support the use of gene drive to control agricultural pests, if the mechanism limited spread.

This public perception is complicated by the fact that the science involved in invasive species eradication is often complex and is not currently well understood by or communicated to the general public. In a survey of the public on gene drive in agriculture, 85% of respondents were not aware of the existence of gene drive technology prior to receiving the survey

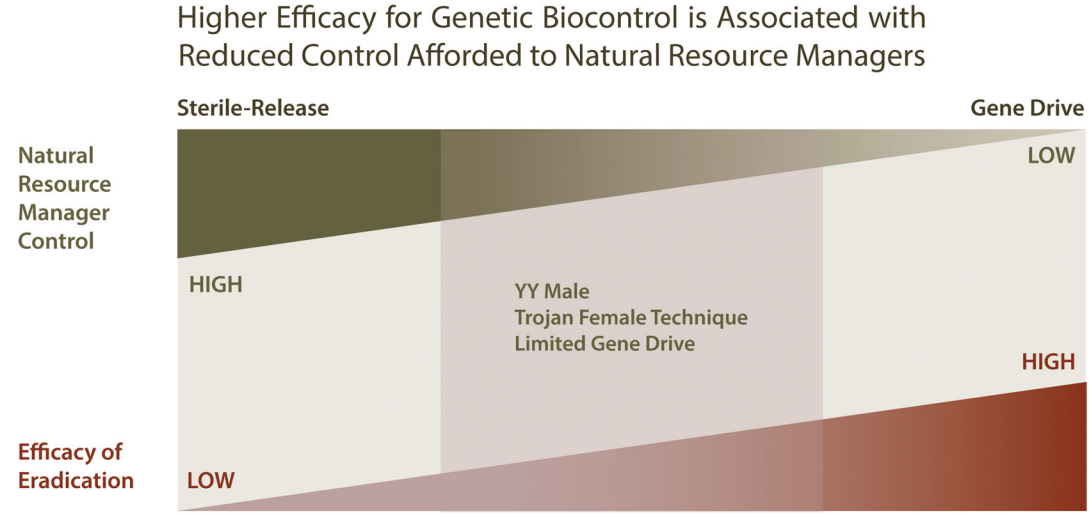

FIGURE 5 | Comparing genetic biocontrol strategies with respect to efficiency and oversight control afforded to natural resource managers. When considering genetic biocontrol strategies, it can be helpful to consider the tradeoff between the efficiency of the genetic method, and the amount of control available to natural resource managers employing the strategy. Genetic biocontrol methods that are potentially the most efficient in eradicating invasive species include gene drives, which are self-sustaining and but thus require much initial from natural resource managers. Although gene drives are highly efficient for eradication, they provide natural resource managers with few options for managing the eradication process. In contrast, sterile-release methods provide an eradication process that is more easily managed, but less efficient, requiring the production and distribution of large numbers of sterile organisms into the environment in order to effect suppression. The YY Male and Limited Gene Drive genetic biocontrol strategies present alternative approaches that are intermediate with respect to efficiency and oversight control.

(Jones et al., 2019). Public attitudes may therefore be determined, not by scientific debate regarding the risks of genetic biocontrol and mitigation strategies used to manage risk, but rather on the outcomes of initial field trials that allow the benefits of invasive species eradication. News articles involving eradication of invasive mice on an island by gene drive or eradication of invasive species could be how the public is introduced and educated on genetic biocontrol technology. In each case the term "genetic biocontrol" will be associated with whatever positive (and negative) outcomes arise from these efforts. The prospects for future applications of genetic biocontrol will thus likely depend on whether initial benefits achieved in these early studies are deemed commensurate with the perceived risk associated with the technology.

CONCLUSION

Eradication of invasive species continues to be a challenge in a variety of ecosystems, ranging from heavily managed agricultural environments to wilderness areas. Tools for effective control are often inefficient and costly, making eradication of invasive species impractical once they have become established. While the potential exists to manage a range of pest with genetic biocontrol, hurdles remain with the implementation of these techniques. For some genetic biocontrol methods, one hurdle is the production of the large numbers of organisms that must be released into the environment. Gene drive, a genetic biocontrol method that is increasingly the focus of public attention, has the potential to spread without the need for sustained human intervention. This theoretically reduces the requirement for mass rearing and release of large numbers of organisms and would allow genetic biocontrol efforts to be applied to remote areas that are difficult to access or lack the infrastructure to support mass rearing efforts. Gene drive could also potentially be used to target invasive species that have become established over very large geographical areas (e.g., Asian carp in the Mississippi River, lionfish in the Caribbean, zebra mussels in the Great Lakes) (Harvey-Samuel et al., 2017). Although gene drive technology offers the potential for efficient, cost-effective, widely applicable genetic biocontrol control for invasive species, it has not yet been deployed in the environment and therefore also presents uncertainties with regard to both efficacy as well as potential unintended effects (Webber et al., 2015; National Academies of Sciences and Medicine, 2016).

Many genetic biocontrol methods, such as YY Males, require a steady influx of individuals with the biocontrol trait into a target population to cause eradication over time. While costly in terms of mass rearing requirements, these mechanisms offer the advantage of allowing for termination of releases if undesirable effects are observed. This can be very attractive to resource managers and to the general public. In contrast, at least some gene drive methods are intended to be self-sustaining. Consequently, the primary concern associated with the use of gene drive as a genetic biocontrol tool for invasive species is that it could spread to a non-target population causing unintended harm (Noble et al., 2018). Because of this and other reasons, the choice of any particular genetic biocontrol method will be informed by a variety of considerations, including the availability and amenability of technologies in the species of interest, the environment where

the invasive species that is the subject of the control is present, public acceptance of the technology being applied and the cost versus benefit of deploying genetic biocontrol as part of a control program targeting any particular species.

Sterile-release and gene drive represent two extremes within a continuum of genetic biocontrol approaches that vary with respect to efficiency vs. control afforded to natural resource managers, while YY Males and the Trojan Female Technique represent an intermediate in this continuum (see **Figure 5**). Sterile-release affords control natural resource managers with a large measure of control and little uncertainty with regard to unintended effects, but for a only a limited number of species and at a high cost with regard to infrastructure requirements. Although gene drive is likely to be more efficacious, require minimal infrastructure and address a greater number of invasive species, it does so at the cost of greater uncertainty of causing unintended environmental effects and providing limited options afforded to natural resource managers for oversight of control. As a compromise between these extremes, YY Male provides a method of genetic biocontrol with modest infrastructure requirements, modest genetic biocontrol efficacy that is applicable to a limited number of species, but with low uncertainty for unintended effects and providing a high measure of control to natural resource managers.

Together with certain technologies, including genetically engineered gene drive constructs or genetically engineered sterility systems genetic biocontrol options exist on a continuum and provide opportunities for the control and potential eradication of invasive species based on our knowledge of inheritance. While the techniques themselves are all unique, they fit under the broader category of genetic biocontrol, and all should be considered in the context of existing biocontrol and invasive species control programs. In considering how best to move forward with the development and deployment of genetic biocontrol methods, it is informative that public surveys on genetic biocontrol techniques reveal, perhaps unsurprisingly, that the general public simply are not educated on the techniques or even aware of them. This suggests that any program intending release of such organisms should also be associated with some form of educational campaign.

We suggest that resource managers, regulators and researchers should work together to ensure that several of these methods be deployed in the future to control invasive species while minimizing the impact on non-target species and the environment.

AUTHOR'S NOTE

The findings and conclusions in this publication have not been formally disseminated by the U.S. Department of Agriculture and should not be construed to represent any agency determination or policy.

AUTHOR CONTRIBUTIONS

JT, LA, SD, ME, OE, NG, TH-S, RM, KO, AP, JS, DS, PT, TS, and AR all made significant contributions to the drafting of the manuscript. KO and AP conducted original data analysis for the manuscript. JT and AR organized the workshop. LA, NG, RM, AR, DS, and PT developed and created the figures. RM and AR lead the editorial efforts to develop the manuscript.

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REVIEW
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Sublethal Endpoints in Non-target Organism Testing for Insect-Active GE Crops

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Historically, genetically engineered (GE) plants that have incorporated genes conferring insect protection have primarily used Cry proteins derived from *Bacillus thuringiensis* (*Bt*) to achieve their insecticidal phenotype. As a result, regulators have developed a level of familiarity and confidence in reviewing plants incorporating these insecticidal proteins. However, new technologies have been developed that produce GE plants that incorporate pest protection by triggering an RNA interference (RNAi) response or proteins other than *Bt* Cry proteins. These technologies have new modes of action. Although the overall assessment paradigm for GE plants is robust, there are ongoing discussions about the appropriate tests and measurement endpoints needed to inform non-target arthropod assessment for technologies that have a different mode of action than the *Bt* Cry proteins. As a result, increasing attention is being paid to the use of sublethal endpoints and their value for environmental risk assessment (ERA). This review focuses on the current status and history of sublethal endpoint use in insect-active GE crops, and evaluates the future use of sublethal endpoints for new and emerging technologies. It builds upon presentations made at the Workshop on Sublethal Endpoints for Non-target Organism Testing for Non-*Bt* GE Crops (Washington DC, USA, 4–5 March 2019), and the discussions of government, academic and industry scientists convened for the purpose of reviewing the progress and status of sublethal endpoint testing in non-target organisms.

Keywords: non-target organisms, sublethal endpoints, *Bt* Cry, GE plants, environmental risk assessment, RNAi

INTRODUCTION

The introduction of insect-resistant GE crops began in the 1990s, with a number of today's crops incorporating *Bt* Cry proteins (Koch et al., 2015; Naranjo et al., 2020). According to the International Service for the Acquisition of Agri-biotech Applications (ISAAA) GM Approval database[1], 85 transformation events involving *Bt* Cry protein expressed in 10 crops received regulatory approval somewhere in the world by the end of 2019. These have been further incorporated into 206 stacks (that combine two or more GE traits) that have received additional regulatory approvals[1]. These approvals have each been accompanied by an ERA that has typically focused on identifying the target range and specificity of the *Bt* Cry proteins using a tiered approach to non-target testing that is very similar to the approach used in the assessment of chemical pesticides (Garcia-Alonso et al., 2006; Romeis et al., 2008; **Figure 1**). Tier-1 testing involves the use of surrogate species tested under worst-case exposure conditions in the laboratory to identify potential hazards to them. Specific endpoints, typically including mortality, are measured in tier-1 tests under conditions of exposure to concentrations, usually several fold higher than concentrations expected in the field (Romeis et al., 2008). In the absence of relevant negative effects in test species at high exposures, a conclusion that the likelihood of adverse ecological effects under realistic conditions is low or negligible can be supported (Romeis et al., 2013b). If negative effects are observed under worst-case conditions, then higher tier studies are conducted to establish if the effect is relevant under more realistic conditions (i.e., lower dose; Rose, 2007; EFSA, 2010; **Figure 1**).

After more than 20 years of use in the field, there is a substantial history associated with GE plants incorporating *Bt* Cry proteins and their safety in the environment (Mendelsohn et al., 2003; Naranjo, 2009; Duan et al., 2010; Center for Environmental Risk Assessment, 2012; Guo et al., 2014; Koch et al., 2016; Romeis et al., 2019). The utility of a tiered approach using mortality as the primary endpoint is supported by experience with *Bt* Cry proteins and by an understanding of the mode of action, target specificity and exposure levels for these proteins. However, new pest control technologies, including non-*Bt* Cry proteins and the use of RNAi, have led to an increased interest in sublethal endpoints testing. This interest is due to several factors, including broader interest in sublethal impacts of chemicals, differences in the mode of action, and the length of time required to observe an effect, and concerns about cumulative or additive effects of multiple stressors in the environment.

While the use of sublethal endpoints is potentially informative for the ERA of non-*Bt* GE plants, there are a number of challenges to implementing this approach. These include the wide variety of potential endpoints from which to choose and difficulties interpreting the relationship between sublethal endpoints in laboratory studies and observable effects in the field. This paper addresses some of these challenges by examining sublethal endpoints in the context of insect-resistant GE plants and considering when inclusion of sublethal endpoints may or may not be warranted in the context of problem formulation for a case-specific ERA. This review has been informed by discussions at a workshop convened by the ILSI Research Foundation (Washington, DC, USA, 4–5 March 2019) that asked participants to consider the relevance of sublethal endpoint testing using a case study approach.

[1]http://www.isaaa.org/gmapprovaldatabase/

NON-TARGET ORGANISM ASSESSMENT

Problem Formulation

The process of identifying and refining the information that will be informative for case-specific ERA is referred to as problem formulation (USEPA, 2016). The mechanics of problem formulation can be described in a number of ways (Hill, 2005; OGTR, 2009; Wolt et al., 2010; Gray, 2012), but the process involves a series of steps that incorporate context for the decision being made, information about the receiving environment and the societal values or protection goals that are identified in relevant laws and regulations. Because these protection goals are often broad, case specific ERA requires the refinement of operational protection goals and the subsequent identification of assessment endpoints, which allow the testing of relevant hypotheses to inform the assessment (Sanvido et al., 2012; Devos et al., 2015). Data are then collected under laboratory, semi-field or field conditions for measurement endpoints that are related to those assessment endpoints (Garcia-Alonso and Raybould, 2014). The advantage of problem formulation is that it provides an explicit rationale for how and why a particular measurement endpoint will be informative to an ERA (**Figure 2**).

For most risk assessments on non-target invertebrates, operational protection goals are reliant on maintaining populations of value or beneficial arthropods that contribute to important ecosystem services (Devos et al., 2015). Among the most relevant have been populations of pollinators, parasitoids, and predators as well as charismatic, protected, threatened, or endangered species, for which an exposure assessment indicates they will have a meaningful exposure to the GE plant. Once the particular species of interest are identified, appropriate surrogate, indicator, or focal species are selected for testing (Rose, 2007; EFSA, 2010).

NTO Study Design

As with any research or regulatory study, the design of an non-target organism (NTO) study must be appropriate for the intended end use of the data. Well-designed early-tier studies are intended to identify any hazards that require further study. Careful consideration, therefore, must be given to ensuring that tests are reliable and especially that false negative results (i.e., failing to identify a hazard) are avoided since they would lead to the release of hazardous material. False positive results, on the other hand, also should be avoided since they might have consequences, even beyond the triggering of additional studies (Romeis et al., 2013a). The first step is to identify appropriate surrogate species (Carstens et al., 2014). These should be chosen based on the representativeness of species

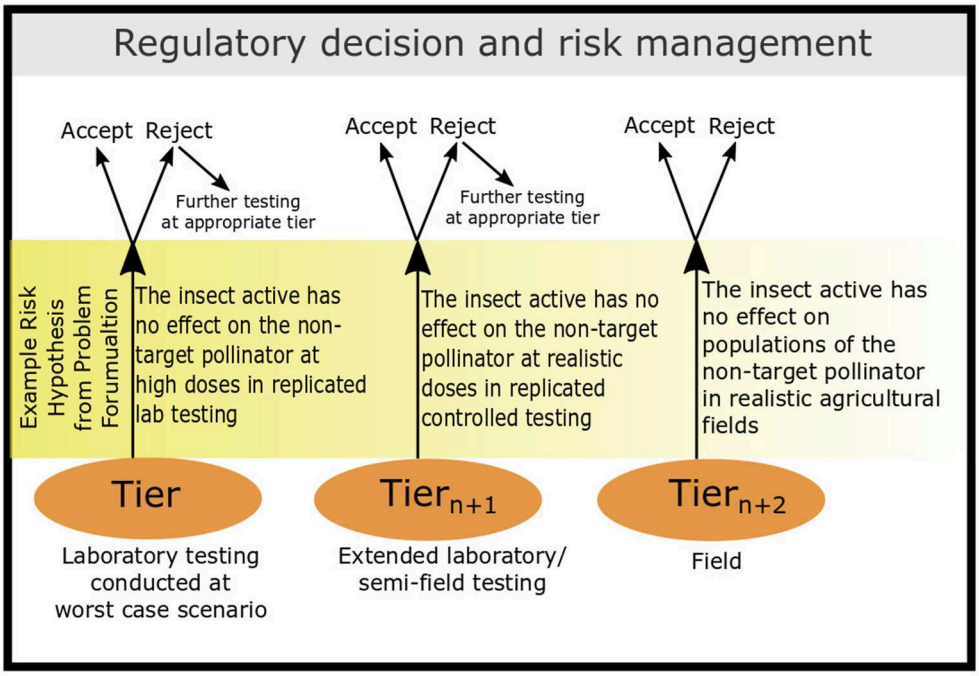

FIGURE 1 | This figure details the tiered structure of testing used during the ERA process. This is hypothesis-based testing, where the initial tests are conducted at doses higher than what is encountered in a natural setting to simulate a worst-case scenario exposure. Example risk hypotheses are provided for NTOs to demonstrate how this structure can be used to test sub-lethal effects. This figure was adapted from Romeis et al. (2008).

of importance in the expected receiving environment, and ideally represent a functional group of interest. As a practical consideration, it is useful to select a surrogate from readily available laboratory reared strains. This provides uniformity to the test and control groups as well as reproducibility of results obtained from multiple laboratories. A surrogate species may additionally be selected based on its sensitivity to the test substance, taking into account what is known about the spectrum of activity of the insecticidal protein. For example, when the surrogate species and the target pests are more closely related phylogenetically, it is more probable that the surrogate species will be sensitive to the test substance (Romeis et al., 2013a). NTO tests should be designed to expose the surrogate to the test substance in a way that maximizes the likelihood of detecting an effect and represents a relevant exposure pathway (e.g., dietary exposure). While juveniles are usually expected to be more susceptible in some species, other species may instead or also have susceptible adults, so it is advantageous to test multiple life stages provided there is a reasonable exposure pathway for those life stages (Romeis et al., 2011, 2013b).

When designing the study protocol, the most expedient approach is to use an already available artificial diet into which both test substances and positive controls can be easily incorporated. This allows the concentrations of the test substance to exceed the expected environmental exposure in order to test levels that exceed worst-case exposures. If an artificial diet is not available, or not suitable for incorporation of the test substance, then GE plant material can be used. However, this may limit the test dose possibilities and reduces the margin of exposure. Furthermore, it might be a challenge to identify the appropriate non-transformed control material.

The use of appropriate controls is a critical component for ensuring that test results are reliable and meaningful (Romeis et al., 2011). When plant material is used as a test substance, near-isogenic lines are preferred as a control in order to eliminate confounding variables in the composition of the materials, unrelated to the GE trait. For NTO studies, negative controls should be designed to mimic the test conditions as closely as possible, and usually use the same test diet with an inert ingredient in place of the test substance. Negative controls also are essential to evaluate if specific *a priori* assay performance criteria were met (e.g., level of acceptable control mortality), to ensure that any observed effects are not due to nutritional inadequacies, failure of the test arthropods to eat the diet or inappropriate conditions in the experimental design for the health of the test arthropods. Similarly, positive controls are recommended to ensure that the test system is functioning as intended. This includes verifying that the test animal is consuming the test substance. To ensure this, having a positive control that closely mimics the mode of action, or at least requires the same route of exposure as the test substance is desirable. Moreover, recombinant insecticidal proteins produced in microorganisms are often used as the test substance in place of plant derived proteins because of the impracticality of purifying a

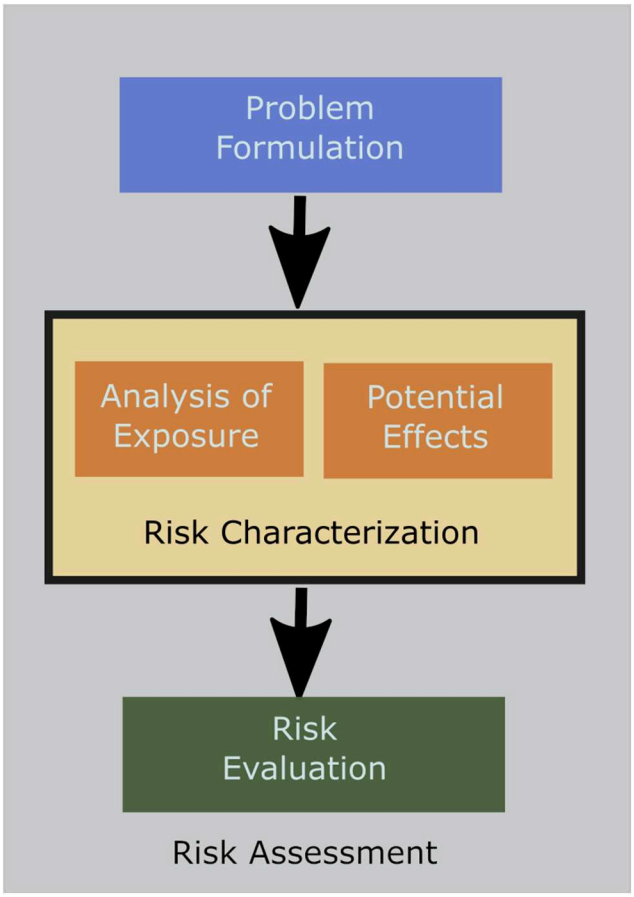

FIGURE 2 | This figure provides details of the broad steps used in the risk assessment process. All risk assessments start with problem formulation to develop the hypotheses that need to be tested. Once problem formulation is completed risk characterization is conducted with analysis of exposure and testing to determine the potential effects of the exposure. Data from the risk characterization studies are used in the risk evaluation process. This figure was adapted from Wolt et al. (2010).

sufficient quantity of protein from the plants. For this reason, it is important to assess the functional and biochemical equivalence of the test substance with the protein expressed by the GE crop (Raybould et al., 2013). Another aspect to consider is the biological activity of the test substance in the diet: a parallel bioassay is usually performed using a target species in order to check that the insecticidal protein is stable and has the expected level of biological activity in the diet. In practice, not every test system is able to meet the definition of an ideal NTO study. However, as long as the methods and protocols are reported accurately and the limitations of the study are understood and explained, these studies should still be considered in a weight-of-evidence approach. As a minimum standard, tier 1 studies should include a worst-case-exposure scenario, confirmation that the test species is exposed to the biologically active test substance, and the use of a negative experimental control (De Schrijver et al., 2016).

Measurement Endpoints in NTO Testing

Historically, the primary and most common measurement endpoint for an early tier laboratory study is mortality. There are a number of reasons for this, including that it is normally unambiguous, easy to measure and has a clear and direct relationship to potential adverse effects on populations of NTOs and ecosystem services they provide. Because it is a common endpoint, there are study designs and methods described for measuring mortality in multiple test systems and for many test species that are validated. Adherence to these standards is often encouraged or required for submitting study results associated with regulatory reviews.

One advantage of using mortality as a study endpoint is that regulatory agencies have developed policies and practices associated with interpreting the results of mortality in NTO studies in regulatory risk assessments. A white paper generated by a panel of experts suggested 50% mortality or a 50% impact on development or weight at the maximum hazard dose in tier 1 studies with insecticidal proteins as a reasonable threshold value for determining if higher tier studies will be informative (Rose, 2007). The European Food Safety Authority (EFSA) recommends a multiplicative effect of 20% in tier 1 laboratory studies in order to trigger additional studies (EFSA, 2010). One criticism of looking at mortality as a measurement endpoint is that it may not be protective for sublethal effects that might impact populations and the ecological services provided by NTOs. While this is certainly true, it is mitigated by achieving a sufficient margin of exposure, margin of safety or other conservative features of NTO study design.

While it is often remarked that mortality is the only endpoint used in in the regulatory risk assessment of *Bt* Cry proteins (Andow and Hilbeck, 2004; Desneux and Bernal, 2010), this is not the case. As shown in **Table 1**, although mortality is the most common measurement endpoint, it is not unusual to see one or more sublethal endpoints recorded for a regulatory study (e.g., larval weight and development time can be collected when conducting lethality testing provided the test is of sufficient duration for the test species to reach developmental milestones). Most regulatory studies on insecticidal proteins have also recorded sublethal endpoints, but these data are not always reported in summaries or subsequent representation of the study. Sublethal endpoints that have been measured in studies for *Bt* Cry proteins include larval and adult weight, developmental timing, fecundity (number of offspring), percent completing adult development and even mobility. What is equally apparent from **Table 1** is that while sublethal endpoints have been measured, sometimes it is not immediately obvious how or why particular data on sublethal endpoints are collected, and there is little consistency in how those endpoints are reported in the literature. This is not unexpected as these measurements are selected and coordinated by individual product managers in the absence of specific data requirements. Standardized and validated test protocols used to assess foliar applications of pesticides published by the International Organization for Biological and Integrated Control of Noxious Animals and Weeds (IOBC) include methods for a total of nine beneficial species (two parasitoids, seven predators) and can inform NTO testing for

GE plants. With the exception of one beetle species (*Aleochara bilineata*, Coleoptera: Staphylinidae), all IOBC protocols assess mortality as an endpoint (Candolfi et al., 2000). In addition, the tests also considered sublethal endpoints, mainly based on reproduction but also food consumption and behavior of the organism.

The selection of measurement endpoints associated with NTO assessment should be guided by a proper problem formulation that incorporates case-specific information pertinent to the assessment. In this way, measurement endpoints are selected that are clearly linked to an assessment endpoint and associated with the risk hypothesis. Risk hypotheses should not be generic, but rather case-specific and informed by what is known about the insect active routes of exposure and its effects on sensitive or target species. Also, prior to the study, the project managers and risk assessors should know specifically how the measurement endpoints will be interpreted and how they will be used in the decision process. If it cannot be clearly articulated how study results will inform the assessment, then the measurement endpoints for the study may not be well-aligned with protection goals and decision-making priorities. Finally, the measurement endpoints should be designed to incorporate the practical realities and limitations associated with available and appropriate relevant test species.

EXPERIENCE WITH SUBLETHAL ENDPOINT TESTING IN NON-*Bt* GE CROPS

The development of novel, non-*Bt* Cry proteins for insect control and of non-protein based methods such as RNAi has been accompanied by an increased interest in assessing sublethal impacts. Publications related to these new technologies demonstrate some of the ways that sublethal endpoints are used.

RNAi and MON87411

The potential use of RNAi for pest control has been widely discussed (Burand and Hunter, 2013) and regulators are considering the use of RNAi to control insect pest in relation to risk assessment (USEPA, 2014; Casacuberta et al., 2015; Roberts et al., 2015). Presently, a single insect protected crop using RNAi as a mechanism has been approved for commercialization. The effect of the RNAi in the target pest, the western corn rootworm (WCR, *Diabrotica virgifera virgifera*), is triggered by the presence of a dsRNA targeting a housekeeping gene. MON87411 targets the WCR *Snf7* gene and its mode of action has been well-characterized (Bolognesi et al., 2012; Ramaseshadri et al., 2013). Uptake of *DvSnf7* RNA generates eventual mortality or severe growth inhibition, which can be observed 5 days after exposure to the test substance. There is also a relationship between exposure duration and eventual growth inhibition at lower doses, but short exposures at high doses are sufficient to induce mortality (Bolognesi et al., 2012). A description of the published ERA for MON87411 expressing *DvSnf7* RNA, includes a description of NTO testing conducted in support of the risk assessment (USEPA, 2015; Bachman et al., 2016). A range of vertebrate tests were conducted, as well as arthropod testing including toxicity assays for seven species of arthropod. In addition to mortality, sublethal endpoints were observed for each species and included measures of time to adulthood, percent adult emergence, adult biomass (weight), and fecundity (number of surviving offspring produced). Tests were conducted with concentrations of the test substance in excess of 10-fold the maximum expected environmental concentration and the duration of the test period was in excess of the time required to see an effect in the target species. The selection of surrogate species took into account the mode of action, considering that coleopterans (beetles) show significantly greater sensitivity to ingested dsRNA than other arthropod orders (Roberts et al., 2015). Because this order of insect includes the target species, WCR, it provides a good illustration of how phylogenetic relationships and an understanding of the mode of action can facilitate the choice of surrogates. Additionally, bioinformatics was used as a complementary tool to perform a screening and to identify potential surrogate species based upon the presence of sequence matches. Sequence alignment between the genome and *DvSnf7* informed the number and type of species tested, focusing on those which were considered most likely to be informative (Bachman et al., 2016).

Non-*Bt* Cry Insecticidal Proteins: IPD072Aa

One example of a non-*Bt* insecticidal protein that has been subject to extensive NTO assessment is IPD072Aa, isolated from *Pseudomonas chlororaphis*, which has activity against WCR (Schellenberger et al., 2016). The IPD072Aa protein has been the subject of bioassays to determine the spectrum of activity in order to inform the NTO risk assessment (Boeckman et al., 2019). As the target pest is a coleopteran, bioassays were conducted with 11 species of Coleoptera, representing four families. Additionally, four species of lepidopteran (moths and butterflies) representing four families in this order were tested. Measurement endpoints included mortality as well as weight for all but one species tested, and time to emergence for two species of ladybird beetles. No observed effects were reported for any of the Lepidoptera species, but both mortality and sublethal effects were observed at varying protein concentrations in some of the tested Coleoptera. In line with best practices for NTO study design (see NTO Study Design above) the criteria for selection of species to characterize the spectrum of activity of IPD072Aa was based on several factors such as the phylogenetic relationship between the species and WCR, established laboratory bioassay methodologies, availability of laboratory reared insects, a known suitable diet and reproducibility of the measurement endpoints (Boeckman et al., 2019).

IPD072Aa has a midgut site of action (SOA) where it targets and disrupts midgut epithelial cells causing breakdown of the epithelial lining in WCR through what appears to be a non-pore forming mechanism (Schellenberger et al., 2016). The ability of IPD072Aa to kill WCR larvae resistant to mCry3A or Cry34Ab1/Cry35Ab1 indicates that its target site differs from those of the *Bt* Cry proteins (Carlson et al., 2019). This knowledge related to its mode of action and the phylogentic relationship between the candidate surrogate species and WCR will guide

TABLE 1 | Laboratory studies with beneficial non-target invertebrates (predators, parasitoids, pollinators) or surrogate species for the soil and aquatic environment to support the regulatory risk assessments of plant expressed insecticidal *Bt* Cry proteins.

Species	Life-stage exposed	Measurement endpoints	References
Predators			
Aleochara bilineata	Adults	Fecundity, offspring survival	Stacey et al., 2006[a]
		Fecundity	Raybould and Vlachos, 2011
Coccinella septempunctata	Larvae	Mortality, development time, adult weight	De Schrijver et al., 2016[b]
	Larvae, adults	Larval mortality, development time, adult mortality	Stacey et al., 2006[a];
		Development time, adult weight, fecundity, fertility	De Schrijver et al., 2016[b]
	Adults	Mortality	Raybould and Vlachos, 2011
Coleomegilla maculata	Larvae	Mortality, development time, adult weight	Duan et al., 2002; Devos et al., 2012[c]; De Schrijver et al., 2016[b]; Bachman et al., 2017
		Mortality, weight	De Schrijver et al., 2016[b]
	Adults	Mortality, adult weight fecundity	Duan et al., 2002
		Mortality	Raybould and Vlachos, 2011; Devos et al., 2012[c]
Hippodamia convergens	Adults	Mortality	Devos et al., 2012[c]; De Schrijver et al., 2016[b]
Poecilus chalcites	Larvae	Mortality, development time, development rate, weight	Duan et al., 2006
Poecilus cupreus	Larvae	Mortality, adult weight	Stacey et al., 2006[a]
		Mortality, Development time, adult weight	De Schrijver et al., 2016[b]
Orius insidiosus	Nymphs	Mortality, percent developing into adults	Stacey et al., 2006; Duan et al., 2008[a]; Bachman et al., 2017
		Mortality	Raybould and Vlachos, 2011; De Schrijver et al., 2016[b]
Orius laevigatus	Nymphs	Mortality, development time	De Schrijver et al., 2016[b]
Chrysoperla carnea	Larvae	Mortality	Raybould and Vlachos, 2011; Devos et al., 2012[c]; De Schrijver et al., 2016[b]
Parasitoids			
Pediobius foveolatus	Adults	Mortality	Bachman et al., 2017
Nasonia vitripennis	Adults	Mortality	Devos et al., 2012[c]; De Schrijver et al., 2016[b]
Pollinator			
Apis mellifera	Larvae	Mortality	Duan et al., 2008; Raybould and Vlachos, 2011; Devos et al., 2012; Bachman et al., 2017[c]
		Mortality, development time	Devos et al., 2012[c]; De Schrijver et al., 2016[b]
		Brood development	Raybould et al., 2007
	Adults	Mortality	Duan et al., 2008; Devos et al., 2012[c]
Soil organism			
Folsomia candida	Juveniles	Mortality, number of offspring	Raybould and Vlachos, 2011; Devos et al., 2012[c]; De Schrijver et al., 2016[b]; Bachman et al., 2017
Aquatic organisms			
Daphnia magna	Juveniles	Mortality	Devos et al., 2012[c]
		Mobility	De Schrijver et al., 2016[b]
Culex quinquefasciatus	Larvae	Mortality	De Schrijver et al., 2016[b]

This table only represents sublethal endpoint data collected as part of a regulatory study and does not present sublethal endpoint data collected as part of solely academic studies.
[a]*Data provided in Stacey et al. (2006) are also listed in Raybould et al. (2007). Raybould et al. (2007) summarizes the data that were submitted to the regulatory authority. Interestingly, for all but one of the organisms tested (O. insidiosus; P. cupreus; and A. bilineata) not all sublethal endpoint measured by Stacey et al. (2006) are also reported by Raybould (2007).*
[b]*De Schrijver et al. (2016) lists unpublished data from regulatory studies.*
[c]*Devos et al. (2012) lists unpublished data from regulatory studies.*

selection of the appropriate surrogate species and measurement endpoints in NTO assays.

Non-Cry Insecticidal Proteins: Vip3A

Vip3A is a *Bt* vegetative insecticidal protein that is active against lepidopteran pests. It has a different mode of action from Cry proteins, and when delivered as a combined treatment it has the potential to delay the evolution of pest resistance to *Bt* crops (Lee et al., 2003). A description of the ERA for MIR162, a maize event expressing the Vip3A protein, has been published (Raybould and Vlachos, 2011). The bioassays were conducted using species representing functional groups of foliar arthropods, soil-dwelling invertebrates, pollinators, wild birds and mammals, aquatic invertebrates and farmed or wild fish (Raybould and Vlachos, 2011). In addition to mortality, sublethal endpoints such as fecundity, weight increase, adult emergence, body weight, and length were recorded for some of the species tested. Depending on the species, worst-case, or conservative maximum expected environmental concentrations were used.

DISCUSSION

Sublethal Endpoints Are Addressed in the Context of Existing Frameworks for NTO Assessment

The rationale for conducting NTO studies in support of regulatory risk assessments is not dependent on whether the studies are designed to measure mortality or sublethal endpoints. Before a study is conducted, the problem formulation process should identify a set of informative tests based on the pathways of environmental exposure, and an identification of relevant taxa and functional groups for which risk should be assessed. Once the appropriate tests are identified, consideration can be given to what sort of measurement endpoints will best inform the assessment. When making these decisions, it is important to keep in mind the practical limitation associated with NTO testing and risk assessment.

Absence of a Single Definitive Test or Sublethal Endpoint

As with other types of testing, there is no single definitive test that can address every possibility of sublethal effects. As is typical of the regulatory risk assessment process, consideration of whether a test is necessary should be done on a case-by-case basis, focusing on the value of the information for reaching conclusions about overall risk. Although flexibility in testing allows sublethal tests to be tailored to specific needs of a chosen test system, non-uniformity in testing procedures can make it difficult to compare results obtained by independent researchers conducting experiments and then reporting sublethal impacts in the literature. These comparisons may provide helpful context for regulatory considerations. The endpoints that have been previously utilized to measure sublethal effects for all types of insect-active GE plants (i.e., weight, growth and developmental time, fecundity) appear to be useful and sufficient for sublethal testing of non-*Bt* Cry insect actives, both protein-based and dsRNA-based.

Effect of the Active on the Target Can Indicate the Utility of Sublethal Endpoints in NTO Testing

Knowledge regarding the mode of action and time to effect, as it relates to the effect of the insect active on the target insect, is instructive for ascertaining whether sublethal endpoint testing is likely warranted in NTOs. For example, if an insect active targets a cellular process that broadly affects growth (e.g., protein synthesis) it may be reasonably expected that collection of data measuring sublethal endpoints such as weight, development time, and reproduction may be warranted. If those endpoints are affected in the target organism, they may also be affected in NTOs. Additionally, the time that it takes for sublethal effects to manifest in the target organism during lethality testing may also be an indication that sublethal endpoint testing is warranted for NTO assessment. If mortality in the target organism is delayed, but indications of mortality are evident earlier due to the onset of sublethal effects such as delayed growth or development, then it may make sense to look for these effects in NTO assessment as well.

Sublethal Endpoints Need to Relate to Ecologically Relevant Assessment Endpoints

Sublethal endpoints that relate to measurable ecologically relevant endpoints will be much easier to interpret than more complicated endpoints. Typically, measurement endpoints for sublethal effects that include development time, growth/weight and reproduction are used for data collection, and these endpoints are readily quantifiable and relatable to assessment endpoints such as population size. While a variety of additional sublethal endpoints reflecting more ambiguous measurements have been reported in the published literature (i.e., feeding behavior and learning performance; Ramirez-Romero et al., 2008), the interpretation of these endpoints with respect to ecological outcomes is challenging.

Practical Considerations for Developing New Test Systems

Any NTO testing is dependent on the existence or the development of a well-characterized and validated test system. Some existing test systems may lend themselves to the collection of sublethal endpoint data, but others may not. Thus, development of new test systems may be required when and if the problem formulation indicates that a particular insect active is likely to cause sublethal effects in relevant NTOs. In either case, before the tests are conducted it is important to assure that the results of the sublethal measurements will be meaningful for risk assessment.

Addressing Knowledge Deficits

For RNAi-based insect actives, bioinformatics will become increasingly important in predicting potential adverse effects

associated with exposure to dsRNA. However, there are currently significant gaps in bioinformatics data for both pest organisms and for ecologically important taxonomic groups, as well as a lack of information on which taxonomic groups are (in)sensitive to environmental RNAi. Narrowing those information gaps will provide a better understanding of the extent to which different NTOs need to be assessed for a case-specific assessment for a particular environmental RNAi.

More sublethal endpoint data are collected than is widely acknowledged. In part, this is, due to the primary status of mortality in testing guidelines and in summary reports or journal publications where sublethal endpoint measurements often are relegated to supplementary material. Finding new means of sharing this information (and improving access to it) is needed in the future to increase the potential usefulness of this information for risk assessments. Similarly, data collected during early characterization of pesticidal proteins may be considered proprietary in nature, presenting a barrier to broad distribution. Whenever possible, however, mechanisms should be encouraged to improve access to this type of information. There are several recent examples where early characterization of pesticidal molecules have been published (Bachman et al., 2013, 2017; Boeckman et al., 2019). However, the research community is encouraged to find new means of storing and disseminating information that often is omitted from peer-reviewed publications but has value as a collective resource in further development of NTO risk assessment methodology.

CONCLUSIONS

NTO testing is conducted in support of regulatory decision making, and therefore must be designed for this purpose, rather than simply to conduct scientifically interesting experiments (Raybould, 2010). The testing associated with any particular insect-active GE crop should be informed by a problem formulation process. Problem formulation takes into account what is known about the insect-active protein, the crop, and the expected interactions between NTOs and the associated insect-active crop, as well as the availability of well-developed test systems that facilitate the interpretation of test results in a regulatory context. The problem formulation process remains fundamentally the same whether the measurement endpoint is mortality or sublethal endpoints. When selecting sublethal endpoints for consideration, a risk hypothesis should link the sublethal endpoint to an assessment endpoint and the associated protection goal. Because most NTOs are protected at the population level and NTO communities at the functional level, typically, sublethal endpoints that are related to growth, development, and reproduction and which can be easily extrapolated to population level effects are most informative. While additional sublethal endpoints might be measurable, they should only be considered for regulatory testing if there is a clear relationship to a protection goal and the results are likely to reduce uncertainties associated with the NTO assessment. A review of recent and past measurement of sublethal endpoints collected to inform regulatory studies of plant incorporated insecticidal *Bt* Cry proteins is summarized in **Table 1**. These data suggest sublethal endpoints for current insect resistant GE crops are observed and measured more routinely than is often claimed in the literature.

A long history of standardization exists that can inform the future of NTO testing. While standard methods are not absolutely required for testing possible sublethal impacts, such studies can be informative for risk assessment. However, for these studies to be informative, there should be a clear understanding of what data are being collected and what is the rationale for collecting them. When published in peer reviewed publications, these sublethal endpoints are often published as supplemental data. If sublethal testing is done, and data are reported, these data should be presented more prominently in research reports. This practice would promote a broader understanding and further discussion of the utility of sublethal endpoints and enhance their usefulness to the risk assessment process.

DISCLOSURE

The information and views are those of the authors as individuals and experts in the field, and do not necessarily represent those of the organizations where they work. Mention of a proprietary product does not constitute an endorsement or a recommendation for its use by Greenlight Biosciences, Inc., Iowa State University, ILSI Research Foundation, Newcastle University, The University of Arizona, University of Kentucky, University of Nebraska-Lincoln, or USDA.

AUTHOR CONTRIBUTIONS

JR, CB, RH, MM, FV, and AR lead the initial drafting of the manuscript. CB, JB, ME, RH, SL, MM, RM, JT, TR, AV, JR, FV, XZ, and AR all made significant contributions to the drafting of the article and developing the table. CB, JB, ME, RH, SL, MM, TR, AV, JR, FV, XZ, and AR attended the ABSTC sponsored workshop. JT organized the ABSTC sponsored workshop. RM, AV, and AR developed and created the figures. RM and AR lead the editorial efforts to develop the manuscript.

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Assessment of Gene Flow to Wild Relatives and Nutritional Composition of Sugarcane in Brazil

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The commercial release of genetically modified organisms (GMO) requires a prior environmental and human/animal health risk assessment. In Brazil, the National Biotechnology Technical Commission (CTNBio) requires a survey of the area of natural occurrence of wild relatives of the GMO in the Brazilian ecosystems to evaluate the possibility of introgressive hybridization between sexually compatible species. Modern sugarcane cultivars, the focus of this study, derive from a series of hybridization and backcrossing events among *Saccharum* species. The so-called "*Saccharum* broad sense" group includes around 40 species from a few genera, including *Erianthus*, found in various tropical regions, particularly South-Eastern Asia. In Brazil, three native species, originally considered to belong to *Erianthus,* were reclassified as *S. angustifolium* (Nees) Trin., *S. asperum* (Nees) Steud., and *S. villosum* Steud., based on inflorescence morphology. Thus, we have investigated the potential occurrence of gene flow among the Brazilian *Saccharum* native species and commercial hybrids as a requisite for GMO commercial release. A comprehensive survey was carried out to map the occurrence of the three native *Saccharum* species in Brazil, concluding that they are sympatric with sugarcane cultivation only from around 14°S southwards, which precludes most Northeastern sugarcane-producing states from undergoing introgression. Based on phenology, we concluded that the Brazilian *Saccharum* species are unable to outcross naturally with commercial sugarcane since the overlap between the flowering periods of sugarcane and the native species is limited. A phylogenomic reconstruction based on the full plastid genome sequence showed that the three native *Saccharum* species are the taxa closest to sugarcane in Brazil, being closer than introduced *Erianthus* or *Miscanthus*. A 2-year study on eight nutritional composition traits of the 20 main sugarcane cultivars cultivated in Brazil was carried out in six environments.

The minimum and maximum values obtained were, in percent: moisture (62.6–82.5); sucrose (9.65–21.76); crude fiber (8.06–21.03); FDN (7.20–20.68); FDA (4.55–16.90); lipids (0.06–1.59); ash (0.08–2.67); and crude protein (0.18–1.18). Besides a considerable amount of genetic variation and plastic responses, many instances of genotype-by-environment interaction were detected.

Keywords: interspecific hybrids, natural hybridization, *Saccharum asperum*, *Saccharum angustifolium*, *Saccharum villosum*, *Saccharum* × *officinarum*, geographic distribution, phylogeny

INTRODUCTION

Genetically modified crops have become a useful tool in agriculture and are able to foster economic development, but they have stimulated public debate since their introduction in the 1990s (Mujjassim et al., 2019). Public acceptance is an important element for the success of a technology, and the consumers' opinion in relation to GMOs is based on ethical concerns and risk perception, because the licensed cultivars contain elements derived from genetically incompatible species, and may contain exogenous antibiotic or herbicide resistance genes of prokaryotic origins. Some of the concerns led 38 countries all over the world, including 19 in Europe, to prohibit officially the cultivation of GM crops, although they allow the import of both human food and animal feed derived from GM plants [International Service for the Acquisition of Agri-biotech Applications (ISAAA), 2017; Mujjassim et al., 2019].

Since the first release of a commercial GM crop, the "FlavrSavr®" tomato, in 1992, the adoption of this new technology has been quick. Until 2015, the main GM crops globally marketed were soybeans (cultivated in 95.9 million ha), maize (58.9 million ha), cotton (24.9 million ha), canola (10.1 million ha), and other minor crops, such as beets, alfalfa, papaya, pumpkin, eggplants, potatoes, apples, sugarcane, and poplar, which together correspond at most to 1.9 million ha [International Service for the Acquisition of Agri-biotech Applications (ISAAA), 2020]. All the main cultivated GM crops are propagated by seeds, facilitating the biosafety regulatory process, for once an event is licensed, the genotype can be introgressed into different focus varieties. For the vegetatively propagated crop species, there is a greater challenge, because the licensing is specific for each transgene insertion, that is, a new commercial licensing is necessary for each transformed cultivar, which becomes a limiting factor for the commercial releases.

In Brazil, sugarcane (*Saccharum* × *officinarum*) is a major crop, with 8.38 million ha planted (Companhia Nacional de Abastecimento, 2019), due to its great efficiency in biomass production and to its high sucrose content in the culms (Bonnett et al., 2008; Cheavegatti-Gianotto et al., 2011). However, the challenges with conventional breeding of this species should always be taken into account, mainly the complex genealogy, the polyploid and aneuploid nature of the highly yielding (in terms of biomass and sucrose content) commercial cultivars (Butterfield et al., 2001).

Due to the intrinsic difficulties of traditional sugarcane breeding, the development of cultivars by genetic modification, including gene editing, offers a great potential as it can overcome some of the limitations (Brinegar et al., 2017; Hilscher et al., 2017; Ricroch et al., 2017; Wang et al., 2017; Cristofoletti et al., 2018; Eriksson et al., 2018; Nerkar et al., 2018; Zhang et al., 2018; Khan et al., 2019). The commercial release of GM cultivars is conditioned to the assessment of biosafety risks (Cheavegatti-Gianotto et al., 2011), to meet the requirements of the national regulatory systems (Jaffe, 2004; Eriksson et al., 2018; Khan et al., 2019).

In Brazil, the National Biotechnology Technical Commission (CTNBio) has approved more than 21 contained field trial releases of GM sugarcane in the environment in the last 2 years. These GM approvals are being tested in the field for insect resistance, glyphosate tolerance, biomass yield increase, and tolerance to abiotic stresses such as water deficit (information obtained from the company AgroBio Brasil). The first Brazilian GM sugarcane cultivars ("CTC 20 Bt," "CTC 9001 Bt," and "CTC 93309-4 Bt," from the Center of Sugarcane Technology [CTC], Piracicaba, SP, Brazil), which are resistant to the sugarcane borer (*Diatraea saccharalis*) have already been approved and released for commercial cultivation (Cheavegatti-Gianotto et al., 2018). A rigorous, multidisciplinary risk assessment process, aiming at the potential impact on the environment and at food safety must be followed before the commercial release of GM cultivars and their progeny occur. The risk analyses related to both environmental and food safety required in Brazil addresses the potential of involuntary gene transfer to related species, which might cause negative effects (Ellstrand, 2003; Anderson and Vicente, 2010; Jong and Rong, 2013). We assumed a logical chain of requirements of different natures that ought to be attended should gene flow take place: (1) Species evolutionarily close to the crop are identified (*the phylogenetic requirement*); these species are the main candidates to involvement in gene flow; (2) The occurrence of the candidate wild species is mapped (*the geographical requirement*). The wild and crop species should be sympatric; (3) The wild and the crop species should flower synchronously (*the temporal requirement*); (4) The wild and the crop species should be reproductively compatible (*the physiological/genetic requirement*), which means that they have to be both sexual, produce viable pollen and the pollen tubes of one species have to be able to deliver the male gametes to the embryo sac of the other, producing a viable embryo; (5) Interspecific reproduction should occur spontaneously in the habitat of the species involved (*the ecological requirement*), which means that pollen is successfully transferred and the species are syntopic. In

addition to gene flow studies, analyses also assess the existence of substantial equivalence between the GMO and its parental organism, in the case of GM species used as food and/or feed, to guarantee that no trait other than the target has been introduced inadvertently.

In the case of genus *Saccharum*, native species (*S. angustifolium*, *S. asperum,* and *S. villosum*) occur in several Brazilian regions and are reported in floristic surveys (Filgueiras and Lerina, 2001; Carporal and Eggers, 2005; Kameyama, 2006). In spite of the economic importance of some of the species of the genus, there still are controversies about their taxonomic circumscription and the overall organization of the taxon (Welker and Longhi-Wagner, 2012). The genus *Saccharum lato sensu* includes species of *Erianthus* Michx., and encompasses *ca*. 40 species (Clayton and Renvoize, 1986). However, some authors consider *Erianthus* as distinct from *Saccharum* (Watson et al., 1992; Soreng et al., 2015) and *Tripidium* (Lloyd Evans et al., 2019; Welker et al., 2019) and *Lasiorachis* (Vorontsova et al., 2019) as separate genera. The phylogenetic analysis performed by Hodkinson et al. (2002) did not find any justification for this division, but these authors did not include all species of *Erianthus* in the study. Here we will adopt the circumscription of *Saccharum* in its wider sense, following Filgueiras and Lerina (2003). In Brazil there are three native species of *Saccharum*, previously classified as *Erianthus*: *S. angustifolium* (Nees) Trin., *S. asperum* (Nees) Steud., and *S. villosum* Steud. (Filgueiras and Welker, 2013). The information about these species, however, is scarce and is normally present only in floristic surveys (Cheavegatti-Gianotto et al., 2011). Grasses are commonly identified on the basis of their floral characters, which may constitute a problem, since the inflorescence does not persist for a long period of the life cycle of the plants. In the genus *Saccharum*, leaf blade morphology and pilosity are also of taxonomic importance (Welker and Longhi-Wagner, 2012), but during some periods the leaves become dry and do not keep their characteristics.

Extensive botanical information about these species is lacking (Cheavegatti-Gianotto et al., 2011). The genetic studies on the genus *Saccharum* (*lato sensu*) are complex because of the evidence of multiple cycles of past polyploidization events and consequent reticulate evolution, often followed by silencing and elimination of duplicated genes. Thus, phylogenetic reconstruction involving the genera close to *Saccharum* or even the *Saccharum* species, and especially the cultivated hybrids (*Saccharum* × *officinarum*) are challenging, especially if nuclear DNA sequences are used (Garsmeur et al., 2018; Zhang et al., 2018; Souza et al., 2019). Phylogenomics based on chloroplast genome (plastome) sequences may be a solution to overcome the difficulties imposed by polyploidy and aneuploidy, both found in the genus *Saccharum* (*lato sensu*), because the plastome is not affected by the ploidy level.

This work had two main objectives. First, to evaluate the potential for gene flow between three Brazilian wild species of *Saccharum*, and Brazilian commercial sugarcane cultivars, based on genetic relatedness estimated by genome-level phylogenies and by the detection of sympatry. The second objective was to establish a nutritional compositional information database of the principal Brazilian commercial sugarcane cultivars grown in different environments (regions and years), which can be compared with other databases. More detailed studies carried out on the part of the authors, related to the degree of overlap among flowering times, to pollen fertility, and sex distribution (of sugarcane), to the geographic distribution and the prediction of ecological niches of the wild and domesticated species are being or will soon be submitted elsewhere and will expand the scope of this study.

MATERIALS AND METHODS

Saccharum Species and Cultivars

The choice of the Brazilian wild species of *Saccharum* for the geographic distribution study was based on their a priori potential for crossing with sugarcane, which is related to their evolutionary closeness to the crop. There are three species indicated by agrostologists as close relatives of sugarcane: *S. angustifolium* (Nees) Trin., *S. asperum* (Nees) Steud., and *S. villosum* Steud. (Filgueiras and Welker, 2013).

For the phylogenetic analysis by Ultra-Barcoding, based on the chloroplast full genome sequence (Kane et al., 2012), total DNA from leaves of the following materials were utilized: the commercial cultivar SP80-3280; two parental species (*S. officinarum*—Muntok Java; *S. spontaneum*—SES 208), collected in the germplasm bank of the Plant Breeding Laboratory, Center of Nuclear Energy in Agriculture (CENA/USP), Piracicaba, SP, Brazil; three Brazilian wild species (*S. angustifolium*; *S. asperum,* and *S. villosum*), collected in the metropolitan region of São Paulo. In addition, *S. bengalensis* ("US4714") and *Miscanthus nepalensis* ("IND82318"), collected from the germplasm bank of the Center of Sugarcane Technology (CTC), Camamu, BA, Brazil, both from the *Saccharum* Complex. For the following accessions, chloroplast genome sequences are available at GenBank (NCBI): cultivars *S.* × *officinarum* "SP80-3280" (accession AE009947.2) and *S.* × *officinarum* "NCo310" (AP006714.1); *S. arundinaceum* "JW630" (LC160130.1), *Miscanthus sacchariflorus* (NC_028720.1), *Miscanthus sinensis* "Niigata 410" (LC160131.1), and *Sorghum bicolor* "BTx623" (CM000760.3).

For the nutritional composition trials, a collection of 20 commercial sugarcane varieties from Syngenta Cultivar Protection, Itápolis, SP, Brazil, and from CTC were used. The criteria for the choice of the cultivars evaluated were the proportion of planted area in Brazil (relevance), maturation time and adaptability to different production environments. The trials were divided according to the maturation period of the cultivars, *viz*., early (eight cultivars: RB855156, RB855453, RB965917, RB966928, CV7231, CTC9, CTC17, and CTC21) and middle/late (12 cultivars: RB92579, RB835054, RB867515, RB965902, IACSP955000, IACSP955094, CV7870, SP81-3250, SP83-2847, CTC4, CTC15, and CTC20), encompassing, thus, genotypes of the main Brazilian sugarcane breeding programs.

Mapping the Species Occurrence

In order to delimit the occurrence of the three Brazilian species of *Saccharum*, a geographic data gathering was conducted both

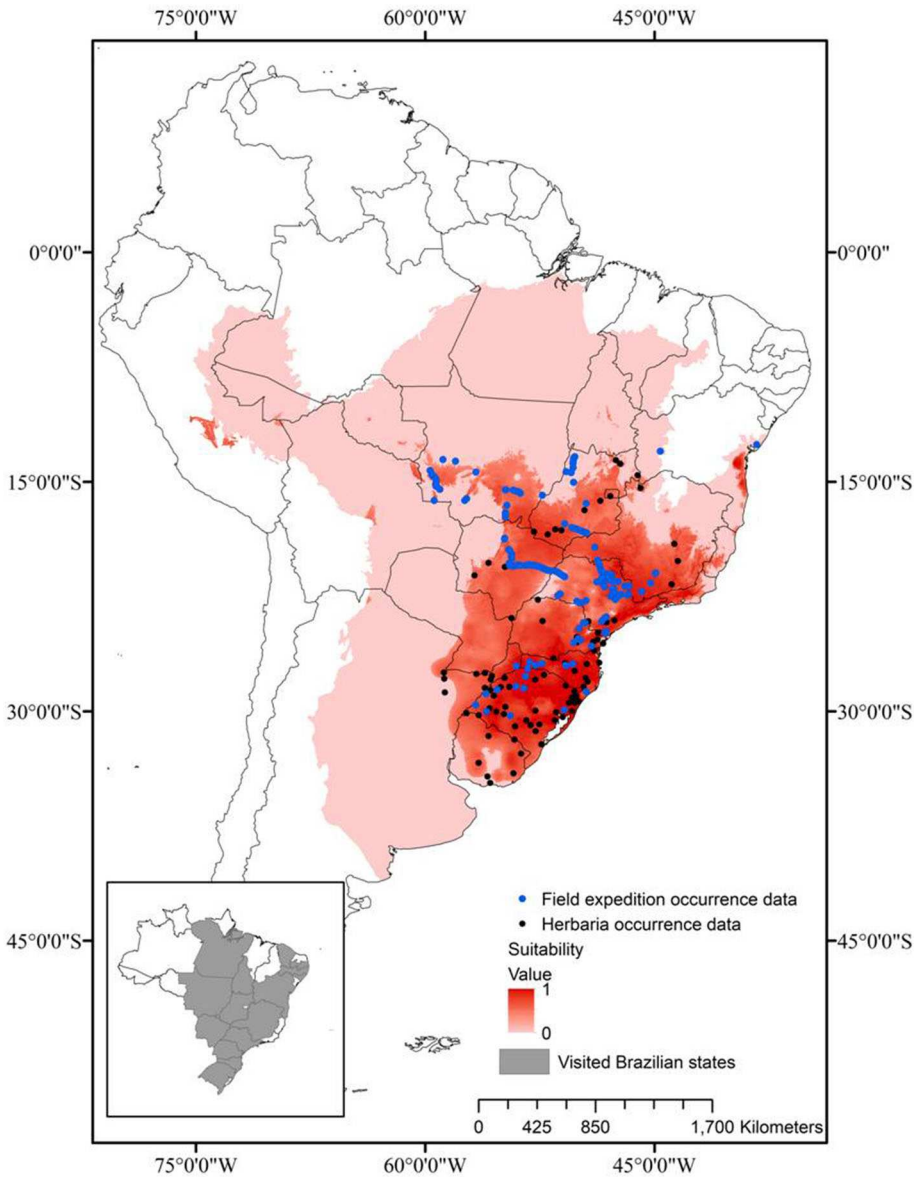

FIGURE 1 | Prediction of the ecological niche of the Brazilian native species of *Saccharum*, based on geographic distribution data (black dots) from previous collecting travels (states visited in gray, in the inset), herbarium data and the literature.

in digitalized/online and non-digitalized international and local herbaria, complemented by field mapping/collecting expeditions in many states of Brazil. Utilizing the main roads of the country, all the regions (South, Southeast, Center-West, North, and Northeast) were visited and a total of 115 sites, with populations of the three species, were mapped (**Figure 1**) and their geographic coordinates were registered with a Garmin GPS 76 (Jaryan et al., 2013).

Distribution Modeling

A niche-prediction model was proposed, based on the raw occurrence data, that helped both describe the biogeography of the species and provide guidance for further collecting efforts, in a iterative way. The Maxent 3.4.1 software (Phillips et al., 2006) was used to generate the distribution models of *Saccharum* species native to Brazil. The GPS coordinates of the 115 points obtained in the collecting expeditions were used as input, together with the points of occurrence obtained from GBIF (Global Biodiversity Information Facility; www.gbif.org). In addition to species occurrence data, environmental data were also used as input for the construction of the distribution model. Data for 19 bioclimatic parameters were downloaded from the Worldclim version 2.0 data portal (www.worldclim.org) for the study area (**Table 1**). These data were downloaded and used in the model (Hijmans et al., 2005), after being converted from "GRID" to "ASCII" format by Arc GIS v. 10.6 (ESRI,

TABLE 1 | The 19 bioclimatic factors tested as model inputs.

Code	Parameter
Bio 1*	Annual mean temperature
Bio 2*	Mean diurnal range
Bio 3	Isothermality
Bio 4	Temperature seasonality
Bio 5	Maximum temperature of warmest month
Bio 6	Minimum temperature of coldest month
Bio 7	Temperature annual range
Bio 8	Mean temperature of wettest quarter
Bio 9	Mean temperature of driest quarter
Bio 10	Mean temperature of warmest quarter
Bio 11	Mean temperature of coldest quarter
Bio 12*	Annual precipitation
Bio 13	Precipitation of wettest month
Bio 14	Precipitation of driest month
Bio 15*	Precipitation seasonality
Bio 16	Precipitation of wettest quarter
Bio 17	Precipitation of driest quarter
Bio 18*	Precipitation of warmest quarter
Bio 19*	Precipitation of coldest quarter

**Indicate variables used as model inputs.*

Redlands, CA, EUA; Scheldeman and Zonneveld, 2010) in order to generate data compatible with MaxEnt. A Pearson correlation test, using the R software (3.4.1; R Core Development Team, 2017), was performed among the 19 bioclimatic variables, with only those variables with correlation coefficients $\leq$0.9 being used for the generation of models, since the autocorrelations between the predictive variables were verified as a recognized source of error (Dormann et al., 2007). Thus, six bioclimatic variables were used to generate the final models (annual mean temperature; mean diurnal range; annual precipitation; precipitation seasonality; precipitation of warmest quarter and precipitation of coldest quarter).

The parameters utilized in the construction of the Species Distribution Models were: convergence threshold of $1e^{-5}$, 500 iterations and 10,000 background points. Each model was subjected to ten repetitions, validated by the bootstrap method. The presence points selected for the generation of the model (70% of total) were partitioned again into two groups, 70% of the occurrence points having been used for training the model, and the remaining 30%, for its internal test. The models were evaluated with the AUC (Area Under the Curve) index. The omission values and the *p*-value were utilized for three cutting thresholds: the 10-percentile training presence Clog-log threshold, the Maximum test sensitivity plus specificity Clog-log threshold and the Minimum training presence Clog-log threshold. The threshold with the least omission values was chosen for the final model. The contribution of the six variables to the final model was tested with the jackknife method. Response curves were generated for the two variables that contributed most to the model.

Phylogenetic Analysis: DNA Extraction

Total DNA was extracted from fresh leaf tissue, with the DNeasy Plant Mini Kit (Qiagen, Valencia, California, EUA). The quality of genomic DNA was evaluated by electrophoresis in 0.8% agarose gel stained with SYBR gold (Invitrogen, Molecular Probes, Eugene, Oregon, USA). DNA concentration was determined by fluorometry (DyNA Quant 2000 Fluorometer, Amersham Biosciences, Buckinghamshire, UK) and with a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, USA).

Phylogenetic Analysis: Chloroplast DNA Sequencing

DNA samples were fragmented by sonication (400- to 500-bp), and the fragments were ligated with adaptors using the Nextera DNA sample preparation kit (Illumina). The chloroplast genomes of *S. angustifolium, S. asperum,* and *S. villosum,* the cultivar SP80-3280, the parental species *S. officinarum* (cv. Muntok Java) and *S. spontaneum* (accession "SES208"), as well as *Miscanthus nepalensis* ("IND82318"), and *S. bengalensis* ("US4714") were sequenced with an Illumina HISEQ2500 platform (Atherton et al., 2010; Nah et al., 2015; Daniell et al., 2016; Dierckxsens et al., 2017), with DNA Single Read or Paired End, Module HIGH OUTPUT—Paired End 2 × 100 pb, and a 100-million-read cover per library, in the Central Laboratory of High-Performance Technologies in Life Sciences (LaCTAD) of State University of Campinas (UNICAMP), Campinas, SP, Brazil. The sequences of the other species were obtained from the Internet databases. With these sequences, it was possible to assemble the plastid genome of part of those species that compose the genus *Saccharum*, including the three Brazilian *Saccharum* species in the phylogenomic analysis. The *Saccharum* plastid genome was assembled based on the published sequence of the "NCo310" (GenBank AP006714.1) sugarcane hybrid (Asano et al., 2004). At the end, the total cover was 14 times as long as the chloroplast genome length.

Phylogenetic Analysis: Phylogeny Reconstruction

The amino acid sequences codified by all the genes present in 13 chloroplast genomes were concatenated and then aligned according to the standard configuration of the Muscle Alignment tool in Geneious R9.1 (Kearse et al., 2012). The amino acid substitution model Blosum62+I+G+F was indicated as the most adequate by the software ProtTest (Abascal et al., 2005) and a maximum likelihood phylogenetic tree was generated with 1,000 bootstrap repeats by RAxML v. 7.7.8 (Stamatakis, 2006). The analyses involving the structural similarities among chloroplast genomes of the "*Saccharum* broad sense" and their phylogenetic relationships utilized the sorghum cultivar BTx623 as an outgroup.

Nutritional Composition: Field Experiment

The cultivar trials were performed in six environments, which represent the main sugarcane cultivation regions in Brazil: Conchal [22°24′S; 47°06′W; 591 m above sea level (asl), State of São Paulo], Jaboticabal (21°16′S; 48°23′W; 615 m asl, State of São Paulo), Taciba (22° 23′S; 51°17′W; 416 m asl, State of São

Paulo), Rolândia (23°18′S; 51°22′W; 730 m asl, State of Paraná), Montividiu (17°26′S; 51°10′W; 821 m asl, State of Goiás), and Carpina (07°35′S; 34°15′W; 184 m asl, State of Pernambuco). The climate is classified as Aw in Jaboticabal, Montividiu and Carpina, Cfa in Taciba and Rolândia and Cwa in Conchal, according to the Köppen scale (Köppen, 1936; Kottek et al., 2006). In each environment, the experiment was performed in a randomized blocks design, with three replications. The experimental plots consisted of two parallel ranks 3 m long and 1.4 m apart. The weed, fertilizer, and pest management were done according to local commercial agricultural practice. The experiments were set up in 2014 and there were two annually harvested crops: first-year crop (2015) and first-ratoon crop (2016). Each sample collected for the nutritional and technological composition assessments was made of 10 entire culms, including their culm tips.

Nutritional Composition: Chemical Analyses

The analyses were performed at the Technological Analyses and Simulation Laboratory (LAST) of the Agricultural Sciences Center, Federal University of São Carlos, in Araras, State of São Paulo, Brazil. Sample composition was analyzed according to the recommendations of the Organization for Economic Co-operation and Development (OECD) (2011) for sugarcane, which has sugars as its main derived product. However, the OECD recommends that some sugarcane constituents be measured in entire culms, including the leaves. The culms had their composition analyzed in terms of: moisture [AOAC (Association of Official Analytical Chemists, https://www.aoac.org) 935.29], crude protein (AOAC 2001.11), fat ether extract (lipids) (AOAC 2003.06), crude fiber (Fiber % Cana Tanimoto, Tanimoto method, ABNT NBR16225), fiber in neutral detergent—FND (Ankom method 13), fiber in acid detergent—FAD (Ankom method 12), ash (AOAC 942.05), and sucrose (Pol % Cana Tanimoto, Tanimoto method ICUMSA, method GS5/7-28, 2013). To summarize the sugarcane nutritional composition essays, descriptive statistics, and graphical procedures were performed. For each trait, minimum, maximum, average, confidence interval for average at 95%, and standard deviation of the mean were calculated. Also, limits defined by three times the standard deviation from the mean were calculated to infer the range that encompasses 99% of the data. In order to better understand the data distribution, skewness, and kurtosis were calculated using the package *agricolae* (version 1.3.1) and the graphical representation was done using the package *ggplot2* (version 3.2.1), both run on *R package* (3.6.1; R Core Development Team, 2017).

RESULTS AND DISCUSSION

Occurrence of the Brazilian Species and Niche Prediction

The Brazilian species of *Saccharum* have a regional distribution: *S. angustifolium* (Nees) Trin. occurs in the Southeast and South regions of Brazil, *S. asperum* (Nees) Steud. occurs from the Center-West to the South and *S. villosum* Steud., the most widely distributed, is present from the Northeast to the South (Filgueiras and Welker, 2013). The distribution model that predicts the habitat and the niche of a species depends on the refinement of the variables and the validation tests, but these frequently present distortions (Phillips et al., 2006; Kamyo and Asanok, 2019).

In this study, the six variables most adequate for the determination of the distribution model of the three Brazilian native *Saccharum* species were: (a) annual mean temperature (BIO01); (b) mean diurnal range (BIO02); (c) annual precipitation (BIO12); (d) precipitation seasonality (BIO15); (e) precipitation of warmest quarter (BIO18); (f) precipitation of coldest quarter (BIO19). The climatic patterns establish the distribution limits of the plant taxa at a regional-global level (Shimwell et al., 1982; Woodward, 1987; Prentice, 1992; Taylor and Hamilton, 1994). The most important variables for the construction of the distribution model of the Brazilian native *Saccharum* species were the average annual temperature and the annual rainfall, which together explained 74.3% of the species distribution. These results indicate that rainfall has a crucial role in the distribution of these species, especially because they grow mainly in wetlands of warm regions. Similar results were found, for instance, for the distribution model of *Dipterocarpus alatus* in central Thailand (Kamyo and Asanok, 2019).

The "Area Under Curve" (AUC) Analysis

The model for the Brazilian *Saccharum* species had an AUC of 0.8586 (±0.019). The cutting threshold chosen was the 10th-percentile training presence threshold, since this threshold gave the best results when the balance between omission and overprediction errors was considered. An AUC value of 0.50 indicates that the model should be considered random and a bad predictor, while a value of 1.00 represents excellent precision (Swets, 1988).

The results of the distribution model must be rigorously assessed, because the ecological niche of a species covers an area wider than the geographic zone the species occupies and not all the suitable areas are inhabited (Kamyo and Asanok, 2019). The populations collected in great part of the country, mentioned in Materials and Methods, were utilized in the validation of the distribution model.

Based on the information collected, the suitability threshold of the distribution model was 0.31 and the omission percentage was 9.47%. The distribution model generated by MaxEnt 3.4.1 was highly satisfactory, indicating that 40.1% of the sampling points are located within an area of high suitability ($x > 0.75$), 43.5% have $0.75 > x \geq 0.50$ and only 16.4% are located in unsuitable areas, with $0.5 > x \geq 0.31$. As a contrasting example, Kamyo and Asanok (2019) report for *D. alatus* that, for an area of 53,483 km^2, only 5.84% (704.27 km^2) were highly suitable, 14.59% (1,757.37 km^2) was suitable, 24.83% (2,991.10 km^2) was moderately suitable and 54.72% (6,592.02 km^2) was unsuitable for the species *D. alatus*.

On the basis of both the distribution model of the three native species and the mapping expeditions, it was evident that the wild populations are sympatric in relation to sugarcane only south of the parallel 14°S, which excludes most of the sugarcane

cultivation area in the Northeast, significantly reducing the possibility of introgression.

During the mapping travels throughout Brazil, other kinds of information were gathered, such as the existence of three categories of population, according to size, and stability. First, species may form large, stable populations in humid environments, near brooks, and rivers. The second type refers to small populations, with just a few dozens of individuals, and occupies suboptimal or relatively unfavorable environments, generally disturbed by humans and unstable, where the original vegetation has been partly or totally removed. Thus, the three species may all be classified as invasive, and display putative adaptations for this condition, such as the trichomes on the spikelets and the reproductive system. The third type of population is composed sometimes by one or two individuals, or a few more, and frequently they are very isolated from the larger populations, sometimes settling in suburban zones. They can grow even on ravines or other disturbed terrain, generally in the crevices that can retain rain water for longer.

There is, thus, a high probability that the population dynamics of these wild species fit the classic source-sink model (Pulliam, 1988; for a recent application, see Seipel et al., 2016), where seeds of the central, stable populations, the "sources," disperse over long distances and found a great many unstable populations, the "sinks," that receive migrants regularly, although they have high mortality and are unable to conserve their numbers by themselves. The possibility cannot be discarded that the secondary populations go extinct frequently and are constantly refounded.

The Brazilian wild species of *Saccharum* do not reproduce by cross-pollination (manuscript in preparation), although it is not yet known whether they are autogamous or agamospermic. Both hypotheses will be tested by progeny analysis in a subsequent study. The seeds of the Brazilian *Saccharum* species are formed very early, when the inflorescence is still deep inside the rolled flag leaf; the flowers are very small. Curiously, the seeds are not dormant, an atypical characteristic for an invasive plant, for which dormancy (dispersal through time) is very advantageous (Leverett and Shaw, 2019). Because the Brazilian wild *Saccharum* plants do not have vegetative propagation mechanisms, such as stolons or rhizomes, they depend exclusively on seeds for colonizing new areas.

Phylogenomics

Many wild species of *Saccharum* relatives, including the Brazilian wild species, are allopolyploid (Welker et al., 2015). The three Brazilian *Saccharum* wild species are distinct species; however, there is evidence that natural hybrids between *S. angustifolium* and *S. villosum* may occur (Filgueiras and Welker, 2013), which might be explained by local chasmogamous mutants, phenotypic plasticity or even natural intraspecific variation. Phylogenomics based on whole plastomes allowed us to show the relationships between species and in the future, as we add infraspecific taxa, it may allow us to include individual populations, interspecific hybrids and geographic races as well, in order to assist in the characterization and conservation of the three species.

The *Saccharum* plastid genome sizes ranged from 141,182 bp (*S. asperum*) to 141,869 bp (*S. bengalensis*—US4714), and all the genomes presented typical circular structures, with two-inverted repeat sequences (all the chloroplast genomes sequenced are in **Supplementary Materials 1–8**). The number of Single Nucleotide Polymorphisms in relation to the plastid reference genome of "NCo310" ranged from three (*S. bengalensis*—US4714) to 355 bp (*Miscanthus nepalensis*—IND82318). The number of SNPs was 96 for *S. angustifolium*, 197 for *S. villosum,* and 207 for *S. asperum*.

Gene number in the plastid genome was the least variable component (199 in *S. villosum*, to 204 in *S. asperum*; *S. angustifolium* has 201). GC content also varied little, from 38.3% in *S. angustifolium* to 38.5% in *S. bengalensis*—US4714). These values were similar to those of other Panicoideae, including *S. officinarum* (Asano et al., 2004), *Miscanthus sinensis* (Nah et al., 2015), *Sorghum bicolor* (Saski et al., 2007), *Erianthus arundinaceus* and *Miscanthus sinensis* (Tsuruta et al., 2017).

Comparison of the Chloroplast Genome of *"Saccharum* Broad sense" and *Sorghum*

The chloroplast genomes of the "*Saccharum* broad sense" and of the outgroup *Sorghum bicolor* cv "BTx623" (GenBank #CM000760.3) were aligned (**Figure 2**). The Maximum Likelihood (ML) analysis resulted in a single tree. From the nine nodes, six have bootstrap support values of 100% (**Figure 2**). The Maximum Parsimony analysis generated one single tree, and both the ML and the MP trees have a similar topology, mostly congruent with the published grass trees based on complete chloroplast genomes (Young et al., 2011; Wu and Ge, 2012; Gao et al., 2014; Lózsa et al., 2015; Tsuruta et al., 2017).

The Brazilian wild *Saccharum* species appeared as closely related to the other *Saccharum* species, and are the taxa genetically closest to *S. officinarum*, *S.* × *officinarum,* and *Miscanthus*. This result disagrees with the scheme proposed for the evolutionary history of the hybrids (Ferrari, 2010). Our results also differ from those of Sobral et al. (1994) who, based on a study on 32 genotypes of the *Saccharum* complex using phylogenetic analysis of chloroplast restriction enzyme site mutations, showed that *Erianthus* diverged from other lineages early in the evolution of subtribe Saccharinae. The result also differs from those found by Tsuruta et al. (2017) that showed that the *S. bicolor* chloroplast genome is more closely related to that of *Saccharum* than to that of *Erianthus*. Discrepancies in phylogenies are expected whenever different materials and methods are used. In the cases above, different subsets of Saccharinae species were compared and different techniques were used to generate characters (chromosome morphology, restriction sites, whole chloroplast genome). However, it is noteworthy that in our study, the three Brazilian native species of *Saccharum* were compared to other species close to sugarcane and were found to be the closest, excepting naturally one of the ancestors of the crop.

Our study supports that the Brazilian wild species of *Saccharum* are the Brazilian Saccharinae most closely related to

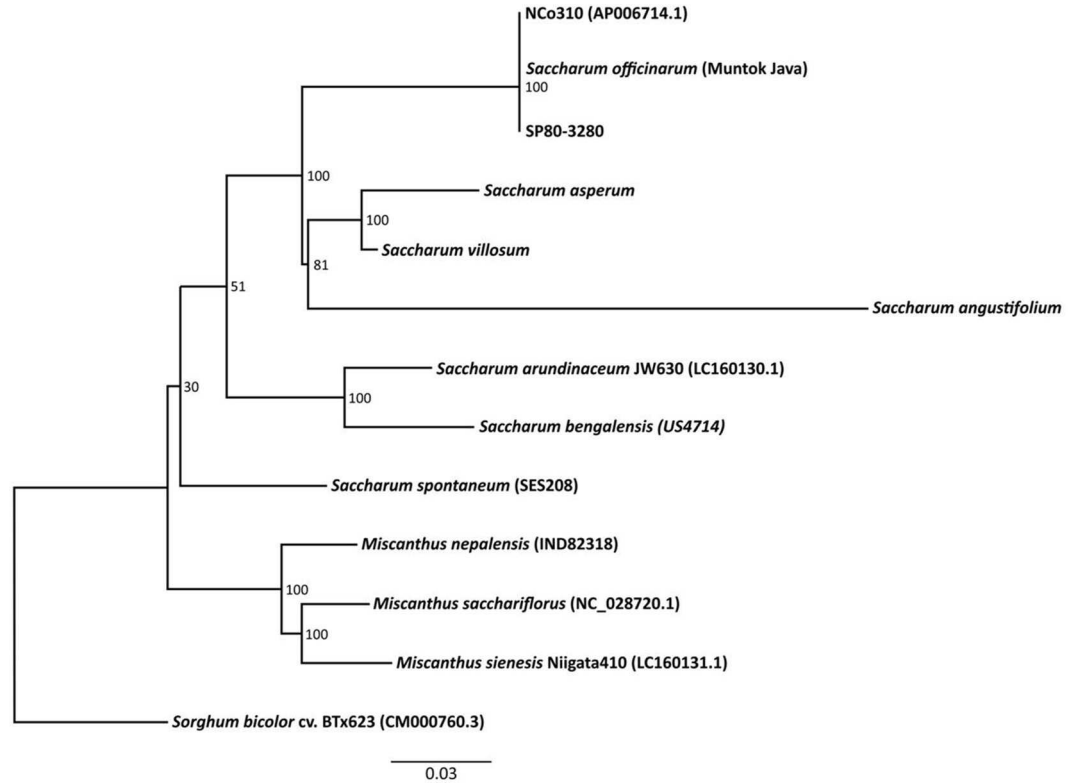

FIGURE 2 | Phylogenetic analysis of 11 species and two sugarcane hybrids, including three *Miscanthus* species that are included in the *Saccharum* complex of wild relatives and *Sorghum bicolor* as an outgroup.

sugarcane, which supports our decision to include these species in this study.

Nutritional Composition

The concept of substantial equivalence was recognized by Organization for Economic Co-operation and Development (OECD) (1993) to ensure that new foods derived from biotechnology be as safe as their conventional counterpart [Organization for Economic Co-operation and Development (OECD), 2011]. This concept was then enhanced through the Codex Alimentarius Commission [founded by the Food and Agriculture Organization, of the United Nations (FAO) and the World Health Organization (WHO)], that developed food standards, guidelines, codes of practice, and other relevant documents under the FAO-WHO Food Standards Programme. In the specific case of sugarcane, the OECD recommends that a new cultivar be analyzed in relation to its contents of main components (humidity, raw protein, lipids, ash, fibers, and sucrose) [Organization for Economic Co-operation and Development (OECD), 2011]. There is local literature on the topic (Azevêdo et al., 2003; Santos et al., 2006; Anjos et al., 2007), but most of the studies are about the use of sugarcane as silage and, more recently, about the release of transgenic cultivars (Gianotto et al., 2019). Nowadays there is no base-line information on nutritional composition of Brazilian sugarcane cultivars, as is required and recommended by the OECD. When we compare the results of the present study with the values previously published by Organization for Economic Co-operation and Development (OECD) (2011), some differences in the minimum and maximum values were identified in, for instance, traits associated with fiber, such as crude fiber (8.06–21.03, our study) vs. (22.7–35.9, OECD), FDN (7.20–20.68, our study) vs. (39.4–77.6, OECD), FDA (4.55–16.90, our study) vs. (24.3–54.4, OECD). Differences in value ranges were also observed for lipids (0.06–1.59, our study) vs. (0.8–1.3, OECD), ash (0.08–2.67, our study) vs. (1.2–6.2, OECD), and crude protein (0.18–1.18, our study) vs. (1.8–4.1, OECD) (the nutritional composition data can be found in **Supplementary Material 9**). These variations may be due to different environmental conditions, genetic background and interference of genotype × environment (G × E). These results highlight the importance of developing databases of percent nutritional composition obtained with cultivation conditions found in Brazil so that the phenotypic ranges observed can serve as comparative values when GM cultivars are assessed there. This reinforces that substantial equivalence assessments should be performed considering databases obtained from sites as close as possible to those where the GMO is to be used.

One of the priority points in substantial equivalence studies is the possible interference of genotype × environment (G × E), which is frequently an important source of variation in sugarcane cultivars observed in many breeding programs all

TABLE 2 | Descriptive statistics for sugarcane nutritional composition traits, as well as skewness and kurtosis estimates.

Traits	Min	Max	Average	Confidence interval**		$\bar{x} \pm 3 \times$ sd ***		Skewness	Kurtosis
			(SEM*)	Lower	Upper	Lower	Upper		
Moisture	62.60	82.50	70.37 (0.11)	70.08	70.66	61.47	79.27	0.31	0.24
Sucrose	9.65	21.76	16.39 (0.07)	16.19	16.59	10.25	22.53	−0.57	0.27
Crude fiber	8.06	21.03	13.72 (0.08)	13.49	13.95	6.62	20.82	0.16	−0.25
FDN	7.20	20.68	13.15 (0.07)	12.95	13.35	6.81	19.49	0.45	0.32
FDA	4.55	16.90	8.58 (0.05)	8.43	8.73	3.90	13.26	1.08	2.51
Lipids	0.06	1.59	0.53 (0.0)	0.51	0.55	0.00	1.06	1.62	4.87
Ash	0.08	2.67	0.59 (0.01)	0.56	0.62	0.00	1.39	2.70	11.39
Crude protein	0.18	1.18	0.54 (0.0)	0.52	0.55	0.11	0.97	0.45	0.80

SEM: standard error of the mean. Rounded to two decimals.

***Confidence Interval obtained at 99%.*

****Three times the standard deviation from the mean (x), may contain 99% of data.*

over the world and constitutes a complicating factor during the selection of clones (e.g., Kang and Miller, 1984; Milligan et al., 1990; Jackson and Hogarth, 1992; Ramburan et al., 2011; Chen et al., 2012). The differential behavior of genotypes in different environments, i.e., the genotype-by-environment interaction, results in alterations in the genotype ranking in competition trials or a change in values of the differences between genotypes in different localities. In general terms, the G × E interaction corresponds to the differential response of the genotypes to changes in the environments, thus evidencing the dependence between genetic and environmental effects. The importance of the study on G × E interactions is well-recognized (Kumar et al., 2018). In this context, the cultivars exposed to some kind of stress may show a wide range of complex and variable responses which depend on the genotype's inherent sensitivity to stress (Chen et al., 2012).

The information on nutritional composition and other components present in both fresh and processed sugarcane is necessary for the development of programs in many areas, such as nutrition, agriculture, industry and food commerce (Giuntini et al., 2006), as well as for being utilized as reference in biosafety assessments of GM cultivars. Although there are many articles about G × E interactions influencing production variables (tons of sugarcane per hectare, Pol per hectare, etc.), there is no report about G × E interaction assessments in variables associated to the nutritional composition in sugarcane.

In this paper, we show an important effort to build profiles of nutritional composition in Brazilian sugarcane cultivars. For this, we set up an experiment across six representative production environments in Brazil, with two harvests (one harvest per year) and varieties from different genetic backgrounds. We evaluated 720 datapoints (20 varieties evaluated at six locations and 2 years with three replicates) that were summarized in **Table 2** and **Figures 3, 4**.

The raw data for each trait was analyzed using descriptive statistics, such as minimum, maximum, average ($\bar{x}$) and mean confidence interval (**Table 2**). Limits were defined by three times the standard deviation from the mean ($\bar{x} \pm 3 \times$ sd). Values of skewness and kurtosis were also estimated. Essentially, two major kinds of information were inferred for the traits, *viz*., the average and the confidence interval. For example, if one considers moisture, the average was 70.37% with standard error of the mean of 0.11. The confidence interval at 99% varied from 70.08 to 70.66%. Still, the minimum observed was 62.20% and the maximum was 82.50%. To suggest outlier candidates, lower, and upper limits defined by a range of ($\bar{x} \pm 3 \times$ sd) can be used. In this case, values varied from 61.47 to 79.27%, suggesting that the maximum value could be an outlier. The same idea can be applied for others traits, i.e., sucrose content showed average of 16.39 and ($\bar{x} \pm 3 \times$ sd) limits of 10.25 and 22.53; for crude fiber, FDN, FDA, lipids, ash, and crude protein, average values of 13.72 (6.62 and 20.82), 13.15 (6.81 and 19.49), 8.58 (3.90 and 13.26), 0.53 (0.00 and 1.06), 0.59 (0.00 and 1.39), 0.54 (0.11 and 0.97) were estimated, respectively. In general, extreme values (outliers) out of the range ($\bar{x} \pm 3 \times$ sd) have been observed for all traits.

The histograms in **Figure 4** provide a visual aid for overviewing the dataset distribution. Briefly, the data pattern suggested a normal distribution for the eight NC traits. Lipids was the trait with maximum concentration of data around the average. In contrast, crude protein had the wider distribution with the shortest peak for the mode. Asymmetry is also suggested for all variables. The distribution shape and asymmetry were quantified by skewness and kurtosis estimates (**Table 2**). For skewness, values close to zero indicate symmetrical distribution. Here, the trait with the most symmetrical distribution was crude fiber (0.16), followed by moisture (0.31), FDN (0.45), and crude protein (0.45). Ash (2.70), lipids (1.62), and FDA (1.08) were the most positively skewed, i.e., with the majority of the data concentrated on the left. The only trait with negative skewness was sucrose (−0.57). It should be stressed that, along the history of sugarcane breeding, breeding programs have focused on selecting genotypes with increasing ability to accumulate sucrose (Morais et al., 2015; Balsalobre et al., 2016; Kumar et al., 2018). With this in mind and considering that the 20 varieties selected had the ability to yield high sucrose content, a left skewed distribution was expected. On the other hand, moisture and fiber-derived traits were not major focuses for selection, which reflected in traits less skewed. The higher absolute values of

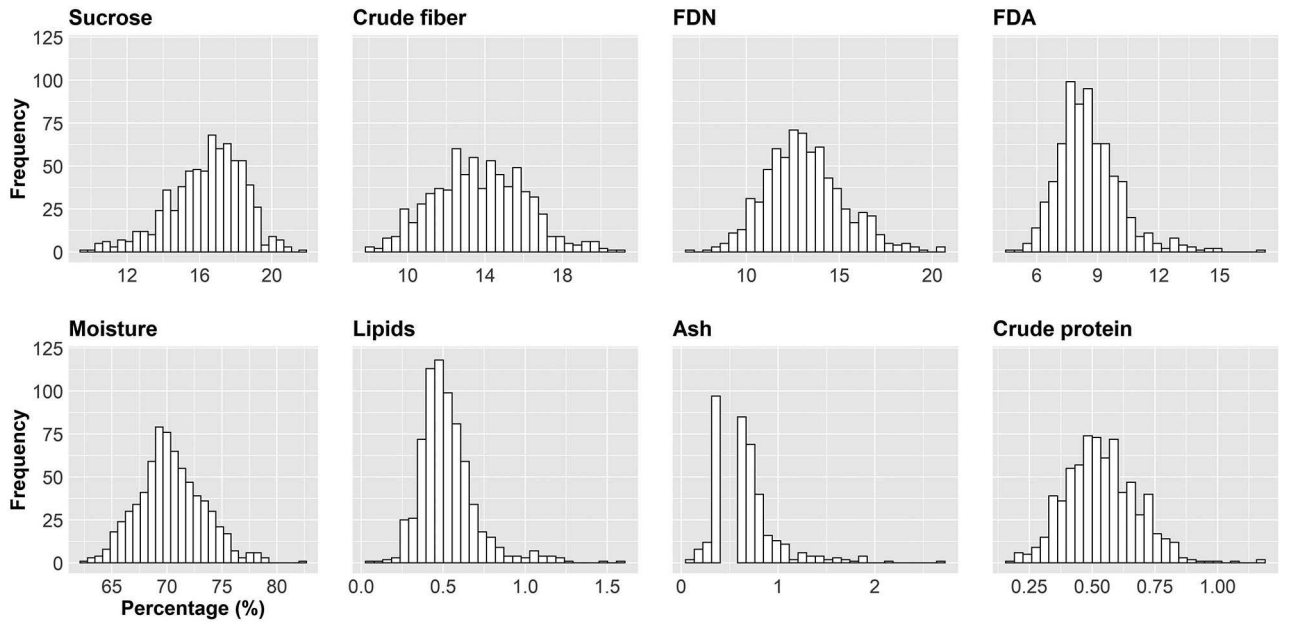

FIGURE 3 | Histogram for the eight sugarcane nutritional composition traits (NC). Axis *x* shows the intervals of the trait indicated in percentage. Axis *y* indicates the absolute frequency (the number of the smallest experimental units) per interval. The smallest experimental unit is each of the replicates of a cultivar/region/year combination.

skewness were obtained for ash and lipids, whose contributions to NC were very small. Kurtosis estimates also provide insights about data variability; e.g., the highest values were found for ash (11.39), lipids (4.87), and FDA (2.51) indicating a concentration toward the mean. On the other hand, the lowest value of kurtosis was found for crude fiber (−0.25) whose distribution was wider than those of the other traits. Moisture (0.24) and sucrose (0.27) showed intermediate values. Considering that our dataset represents the interaction of both Brazilian genetic background and the environmental conditions for sugarcane-producing areas for two crop years, it is possible to infer that the observed range for each trait represents the expected variation for the crop in Brazil and a reference for future studies. However, the extrapolation of these results for different conditions, such as the incorporation of new varieties, more advanced harvest technology or planting in new environments should be done with caution.

Figure 4 shows the percentage of each Nutrition Composition trait but also allows a comparison among environments and among varieties within each trait when the dataset variability is partitioned. For example, when moisture in the whole dataset (**Figure 4A**) was partitioned by environments (**Figure 4B**), the data range slightly changed. It is clear that in five environments (Jaboticabal, Conchal, Rolândia, Taciba, and Montevideo) moisture values tended to overlap but in one single boxplot (Carpina) lower values tended to be more frequent than in other situations. For sucrose content and other traits, minor changes in boxplots can be found among environments. A second partitioning was done by varieties (**Figure 4C**), in which changes in boxplot ranges were fewer than in the partitioning by environments. These results indicate that small ranking changes can be observed when the dataset is partitioned by environments and varieties, validating our results presented in **Table 2**.

An important result was the dispersion of values among nutrition composition traits (**Figure 4**). Here, three groups arise, arranged according to the magnitudes of their values: (a) moisture, appearing in a higher percentage; (b) crude fiber, FDN, FDA, and sucrose appearing with medium percentage values; and (c) lipids, crude protein, and ash, with small percentages. The chemical composition of sugarcane is highly variable, depending on the climatic conditions, the physical, chemical and microbiological properties of the soil, the type of cultivation, the variety, the stage of maturation and age, among other factors. The sugarcane culm can be fractioned into water-insoluble substances—fibers (10–16%)—, and sugarcane juice. On average, 80% of the sugarcane juice consists of water (moisture), and 20% of sugars (e.g., sucrose), lipids, protein, and minerals (Lavanholi, 2010; Kim and Day, 2011; Gianotto et al., 2019).

This work provides information that could be a starting point for studies of substantial equivalence of sugarcane GMOs. The two substances from sugarcane that humans ingest, sugar and ethanol, are produced at high temperatures in the industry, and this minimizes any impact on food safety, because proteins or even nucleic acids would hardly be found in the final product (Joyce et al., 2013). As a smaller-scale example, the new sugarcane GM cultivar CTC91087-6, which expresses the protein Cry1Ac, protecting the plant against the sugarcane borer *(Diatraea saccharalis)*, is substantially equivalent to its conventional counterpart, and its ingestion presents

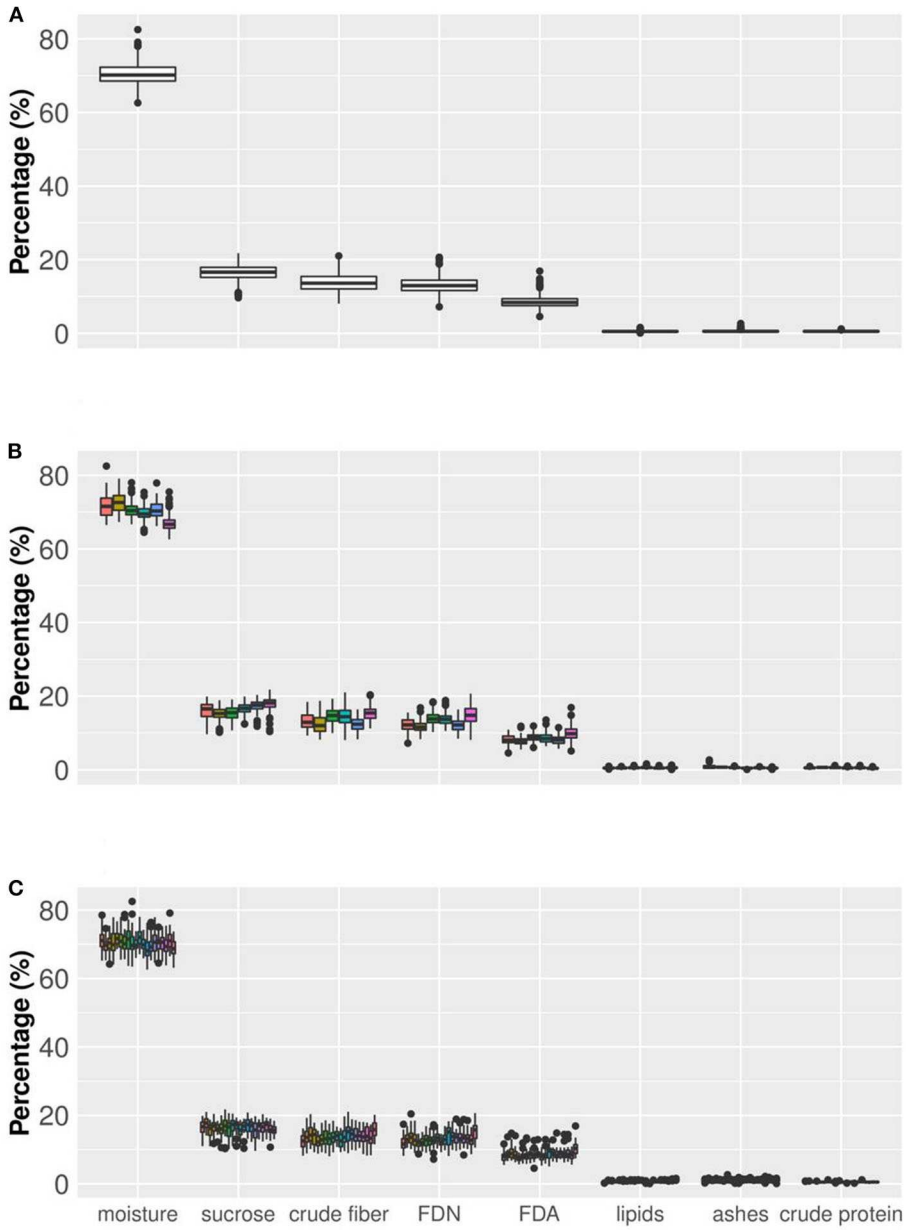

FIGURE 4 | Boxplot of sugarcane nutritional composition traits (axis *x*). Axis *y* indicates the values of the traits, all expressed in percentage. Box edges represent the upper and lower quartile with median value shown as bold line in the middle of the box. **(A)** Boxplot considering all the information for eight nutritional components (NC) (20 varieties and six production environments in two crop years and three replicates). **(B)** Boxplot showing the behavior of NC traits considering six environments (from left to right, Jaboticabal, Conchal, Rolândia, Taciba, Montividiu, and Carpina). **(C)** Boxplot considering the phenotypic variation of the 20 varieties across all production environments (from left to right: CTC04, CTC09, CTC15, CTC17, CTC20, CTC21, CV7231, CV7870, IACSP955000, IACSP955094, RB835054, RB855156, RB855453, RB867515, RB92579, RB965902, RB965917, RB966928, SP813250, and SP832847).

minimal risks to human and animal health (Gianotto et al., 2019).

CONCLUSIONS

The three native wild species of *Saccharum* and the plantations of sugarcane are partially sympatric in Brazil, but the likelihood of introgression is attenuated by their geographical distribution and the reproductive system of the three wild species, which prevents crossing and favors the early formation of seeds still within the rolled flag leaf.

The comparison among the chloroplast genomes provided an important framework for the comprehension of the phylogeny and the evolutionary history of the "*Saccharum* broad sense," where the Brazilian species (*S. angustifolium*, *S. asperum,* and

S. villosum) form a robust monophyletic group, together with *S. officinarum* and the commercial hybrids of sugarcane, but are less closely related to *S. arundinaceus* and *S. spontaneum*.

The nutritional composition studies revealed much genetic variation and plastic responses, and many cases of genotype-by-environment interaction. Thus, there are different responses when a given cultivar is subjected to different production environments and crop years, and the response shapes are different among the cultivars. The information generated will be included in a publicly available database (International Life Sciences Institute—ILSI) to be used in future substantial equivalence studies for genetically modified cultivars.

The three combined results generated indicate that the release of transgenic sugarcane cultivars on Brazilian territory points to no likelihood of gene transfer between sugarcane and its closest wild relatives. In addition, the nutritional composition data related to the 20 top Brazilian sugarcane cultivars are now available for future comparisons.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

EB, MB, ES, and GO conceived of the presented idea. EB, VA, RP, and AF developed the theory to study the chloroplast genome and reconstructed it. EB, GO, MB, MC, ES, and RG were responsible for the nutritional equivalence studies. EB, GO, RS, and IC were responsible for mapping the native *Saccharum* species from Brazil. All authors discussed the results and contributed to the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2020.00598/full#supplementary-material

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Biosafety and Biosecurity in Containment: A Regulatory Overview

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When biosafety for contained use is addressed in international fora and discussions, often the topic is limited to working with genetically modified organisms (GMOs) in facilities such as laboratories, animal facilities, and greenhouses. However, the scope of biosafety in containment encompasses many other types of biological materials, such as human, animal and plant pathogens, nucleic acids, proteins, human samples, animals or plants, or by-products thereof, and overlaps often with the topic of biosecurity. This is also reflected in the regulations that apply for activities with biological materials in contained facilities. The common denominator of these regulations is the focus on protection of people and environment, while applying the key principles of risk assessment and risk management. This review provides an overview of regulatory frameworks for biosafety and biosecurity in containment around the globe, as well as points out overlap with other regulatory frameworks, such as the Nagoya Protocol, or Plant and Animal Health regulations.

Keywords: biosafety, biosecurity, biological material, biological agent, containment, regulations, (bio)risk assessment

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INTRODUCTION

"Biosafety" has multiple accepted definitions depending on the discipline involved (veterinary, food, medical, environmental, or space science), its linguistic roots or even the country in which it is used. Here are a few examples:

- "Safety with respect to the effects of biological research on humans and the environment" (Merriam-Webster, 2019).
- "(Laboratory) biosafety describes the containment principles, technologies, and practices that are implemented to prevent the unintentional exposure to pathogens and toxins, or their accidental release" (WHO, 2006).
- "Principles and practices for the prevention of unintentional release of or accidental exposure to biological agents and toxins" (OIE, 2017).
- "Practices and controls that reduce the risk of unintentional exposure or release of biological materials" (ISO, 2019).
- "The need to protect human health and the environment from the possible adverse effects of the products of modern biotechnology," i.e., the concept of biosafety as described in the introduction of the Cartagena Protocol (SCBD, 2000).

- In terms of outer space, the concept of biosafety is referred to as "planetary protection"—"the practice of protecting solar system bodies (i.e., planets, moons, comets, and asteroids) from contamination by Earth life, and protecting Earth from possible life forms that may be returned from other solar system bodies" (NASA, 2019).

Also the term is sometimes used interchangeably with "biosecurity", although this in itself has many different definitions:

- "Security from exposure to harmful biological agents; also: measures taken to ensure this security" (Merriam-Webster, 2019).
- "(Laboratory) biosecurity describes the protection, control, and accountability for Valuable Biological Materials agents and toxins within laboratories, in order to prevent their loss, theft, misuse, diversion of, unauthorized access, or intentional unauthorized release" (WHO, 2006).
- "A set of management and physical measures designed to reduce the risk of introduction, establishment and spread of animal diseases, infections or infestations to, from and within an animal population" (OIE, 2017).
- (Farm) biosecurity is a "set of measures designed to protect a property from the entry and spread of pests, diseases, and weeds" (AHA/PHA, 2019).
- "Encompasses all policy and regulatory frameworks (including instruments and activities) to manage risks associated with food and agriculture (including relevant environmental risks) including fisheries and forestry and constitutes three sectors (namely food safety, plant life and health, and animal life and health)" (FAO/IPPC, 2019b).
- "Practices and controls that reduce the risk of loss, theft, misuse, diversion of, or intentional unauthorized release of biological materials" (ISO, 2019).
- "The exclusion, eradication, or management of pests and diseases that pose a risk to the economy, environment, cultural and social values, including human health" (MPI, 2016).

Finally, some approaches refer to biorisk management as "coordinated activities to direct and control an organization with regard to biorisk", i.e., "effect of uncertainty expressed by the combination of the consequences of an event (including changes in circumstances) and the associated 'likelihood' of occurrence, where biological material is the source of harm" (ISO, 2019).

As a result of this diversity, "biosafety" and "biosecurity" are frequently used without any agreed definition or scope. The National Research Council (2009) summarizes the difference clearly: "Biosafety is about protecting people from bad 'bugs'; biosecurity is about protecting 'bugs' from bad people".

For the purpose of this article, the following definitions relevant *for contained use* are used:

- **Biosafety**: Containment principles, technologies and practices that are implemented to prevent the unintentional exposure to biological material or their accidental release (adapted from WHO, 2006).
- **Biosecurity**: The protection, control, and accountability for biological agents and toxins within facilities in order to prevent their loss, theft, misuse, diversion, unauthorized access, or intentional unauthorized release (adapted from WHO, 2006).

With regard to "Containment," the concept is generally accepted as "A set of measures including biological containment, practices, safety equipment, and facility safeguards that protect workers, the community and the environment from exposure to and/or unintentional escape of biological material" (adapted from WHO, 2004).

In this paper, we review a selection of objectives that drive the implementation of biosafety and biosecurity in contained environments and how these have been implemented in different parts of the world. Without advocating a specific approach, the review intends to highlight that different systems have been put in place to ensure safety when working with biological material, ranging from voluntary practices to legal requirements.

BIOSAFETY OBJECTIVES

Protecting Workers and the Public Against Hazardous Biological Agents

Concrete references to biosafety practices in microbiology laboratories date from the time of Pasteur and Koch (period of the 1860's–1890's), when, following the first reports of disease in laboratory personnel, the need was identified to implement safety measures in response to potential risks associated with exposure to micro-organisms cultured in the lab. Being able to link certain diseases (e.g., anthrax, tuberculosis, and cholera) to their respective causative agents, Koch decided to handle them in a glazed tabletop box with two openings fitted with oilcloth sleeves. Although far from perfect, the idea of "bio-containment" was born (Berlinger, 2003).

Further research in the domain of laboratory acquired infections (LAIs) in microbiology laboratories contributed considerably to the adoption of protective measures against biological risks (Sulkin and Pike, 1949, 1951; Collins and Grange, 1990). These typically involved a combination of physical containment measures, working practices and personal protective equipment, focusing mainly on occupational safety. Simultaneously, also the US Biological Warfare (BW) program led to innovations in biosafety practices, which were shared at annual conferences starting from 1955 onwards. Although initially restricted to BW laboratories, in the sixties the audience was soon broadened to institutes and agencies involved in health and biomedical research, much to the benefit of their employees and public health (Barbeito and Kruse, 1997; Kruse and Barbeito, 1997a,b).

Protecting Animal and Plant Health

With the development of global trade, the need to prevent and control the introduction and spread of pests of plants and plant products became more important. This led to the foundation of the International Plant Protection Convention (IPPC) in 1951, a multilateral treaty deposited with the Food and Agriculture Organization of the United Nations (FAO). The IPPC

is the standard setting organization for the "Agreement on the Application of Sanitary and Phytosanitary Measures" (the SPS Agreement) of the World Trade Organization (WTO). Specific "International Standards for Phytosanitary Measures" (ISPMs) cover topics such as lists of quarantine organisms, pest risk analysis, or the design of plant quarantine stations, all of which are relevant when applying plant pests under containment in a laboratory or plant growing facility (FAO/IPPC, 2019a).

Similarly, to ensure safe global trade of animals and animal products while avoiding unnecessary obstructions to trade, the World Organization for Animal Health (OIE - Office International des Epizooties, est. 1924) is since 1998 the WTO reference organization for standards relating to animal health and zoonoses (WTO, 2019). The "Terrestrial Animal Health Code" and "Aquatic Animal Health Code" were developed with the aim of assuring the sanitary safety of international trade in terrestrial animals and aquatic animals, respectively, as well as their products. Traditionally addressing animal health and zoonoses only, these codes have been expanded to also cover animal welfare, animal production, and food safety in recent updates (OIE, 2019). As such, they provide concrete guidance for veterinary biosafety aspects of risk management and containment in veterinary research and diagnostic facilities.

Both plant protection and veterinary biosafety not only deal with the actual pathogens, but also define measures to control the vectors of either plant or animal/human diseases, such as arthropods or animal vectors.

Dealing With Uncertainty/Protecting the Environment

Following the discovery of nucleic acids as the central molecules of heredity, the 1970s mark the emergence of a new discipline—molecular biology—with the first experiments with recombinant DNA and cloning being performed in the United States (Jackson et al., 1972). However, in parallel with the discovery of new techniques, questions quickly arose on possible risks associated with these types of experiments, especially because they were largely performed by biochemists less experienced in managing biological risks compared to microbiologists. Following discussions in 1973 (First Asilomar Conference, 1973 and Gordon Conference on Nucleic Acids, 1973), an appeal was made for a voluntary moratorium on experiments involving recombinant DNA until an international conference to assess the potential risks of such experiments was held (Berg et al., 1974). The Second Asilomar Conference (1975), bringing together scientists, legal experts, physicians and journalists adopted two basic principles:

- Containment should be an essential consideration in the experimental design;
- The effectiveness of the containment should match the estimated risk as closely as possible.

In addition, the conference also recommended biological and physical containment barriers as well as the adherence to good microbiological practices, and described a classification of experiments and corresponding containment levels.

One year later, the World Health Organization (WHO, 1976) launched the idea of applying the safety measures successfully implemented in microbiology to contain pathogenic organisms also for recombinant DNA experiments. In response, the National Institutes of Health (NIH) published the first "Guidelines for Research Involving Recombinant DNA Molecules" (NIH, 1976), enabling advances in life science, while promoting the safety of researchers, public and the environment. The "NIH guidelines", revised in 1979, were used as a starting point for many regulations on contained use. Subsequently, some legal frameworks were established to formalize this for specific classes of organisms, referred to as Genetically Modified Organisms (GMOs). As technical progress is moving fast, uncertainty is often used to justify a precautionary approach, further requesting biosafety management for developments of genome editing and synthetic biology.

BIOSECURITY OBJECTIVES

Protection Against Loss, Theft, Misuse, Diversion, or Intentional Release

The WHO Biorisk Management Laboratory Biosecurity Guidance (WHO, 2006) introduced the concept of valuable biological materials (VBM). It is defined as "biological materials that require (according to their owners, users, custodians, caretakers, or regulators) administrative oversight, control, accountability, and specific protective and monitoring measures in laboratories to protect their economic and historical (archival) value, and/or the population from their potential to cause harm". VBM may include pathogens and toxins, as well as non-pathogenic organisms, vaccine strains, foods, GMOs, cell components, genetic elements, and extraterrestrial samples. Next to possible theft, misuse, or intentional release of these VBM, there is also the concern that *bona fide* knowledge obtained from working with these materials in a research setting may at a later timepoint be misused to threaten public and animal health, food security, or the environment, also referred to as "dual use" or "dual use research of concern". Hence, dual use considerations should be an essential part of a biosecurity program.

While laboratory biosafety and biosecurity manage different risks, "they share a common goal: keeping VBM safely and securely inside the areas where they are used and stored" (WHO, 2006).

Preventing Development of Biological Weapons and Addressing Bioterrorism

Following the first World War, marked by the massive use of chemical weapons, several initiatives were undertaken to stop the chemicals arms race and restrict chemical warfare, albeit most of them were restricted to only a few countries (e.g., "Treaty of Versailles, 1919"), or failed to get ratified by all parties [e.g., "Washington Treaty (1922) in Relation to the Use of Submarines and Noxious Gases in Warfare" in 1922]. Negotiations were more successful in Geneva in 1925, with the signing of the "The

Protocol for the Prohibition of the Use in War of Asphyxiating, Poisonous or other Gases, and of Bacteriological Methods of Warfare," usually called the Geneva Protocol (1925). On proposal by the Polish representative, it was the first international agreement that included biological weapons as a separate arms category. However, only the use—and not the development or possession—of chemical and biological weapons was banned. Many signatories reserved the right to retaliate in-kind against states that violated the Protocol, making it *de facto* more of a "no-first-use" agreement. It took until 1972, with the "Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on Their Destruction" (commonly known as the "Biological Weapons Convention" or BWC), before also the development, production, storage, or otherwise acquiring or retaining biological agents and toxins, or related biological weapons or equipment, was prohibited. Exceptions are the application of such materials for prophylactic, protective, and other peaceful purposes (BWC, 1972). A group of 43 State Parties to the BWC has joined forces in the so-called "Australia Group", an informal forum for countries to assist in the implementation of consistent export controls on goods that might contribute to the proliferation of biological or chemical weapons, thereby fulfilling their obligations to both the BWC and the Chemical Weapons Convention (AG, 2020).

ADDRESSING BIOSAFETY AND BIOSECURITY OBJECTIVES IN CONTAINMENT

While the objectives are clearly different, it is evident that biosafety and biosecurity are complementary disciplines that benefit from an aligned approach. It is therefore not surprising that biosafety and biosecurity in containment are often addressed together through a single biorisk management program, ensuring compliance with the requirements and good practices set out in both international guidance documents as well as in the different local legislative frameworks.

International Framework and Guidance Documents

On the international level, different organizations and conventions with relevance for biosafety and biosecurity in containment are established, most of which derive from the United Nations (UN) or operate with it in close cooperation. These include, amongst others, the World Health Organization (WHO), the World Trade Organization (WTO), the World Organization for Animal Health (OIE), the Food and Agriculture Organization (FAO), the International Plant Protection Convention (IPPC), the Convention on Biological Diversity (CBD) and its associated Cartagena and Nagoya Protocols, and the Biological Weapons Convention (BWC), and their historical involvement is described in more detail elsewhere in this article.

These organizations and conventions provide governance on biosafety and biosecurity through a set of internationally accepted reference documents setting out objectives, principles, and requirements. Depending on the document, some of them have a legal basis while others are considered as best practices documents. A non-exhaustive list is given here:

- "WHO Biorisk Management: Laboratory Biosecurity Guidance" WHO/CDS/EPR/2006.6 (WHO, 2006).
- "WHO Laboratory Biosafety Manual: Third edition" WHO/CDS/CSR/LYO/2004.11 (WHO, 2004).
- "WHO International Health Regulations (2005): Third edition" (WHO, 2005) and the associated "Joint External Evaluation (JEE) tool" (WHO, 2016).
- "ISO 35001:2019: Biorisk management for laboratories and other related organizations" (ISO, 2019).
- "OIE Terrestrial Animal Health Code" ("Terrestrial Code"), 28th Ed., 2019.
- "OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals" ("Terrestrial Manual"), 8th Ed., 2018.
- "OIE Aquatic Animal Health Code" ("Aquatic Code"), 22nd Ed., 2019.
- "OIE Manual of Diagnostic Tests for Aquatic Animals" ("Aquatic Manual"), 7th Ed., 2016.
- "IPPC Design and operation of post-entry quarantine stations for plants" ("ISPM 34"), 2016.
- "NIH Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules" ("NIH Guidelines"), April 2019.
- Biosafety in Microbiological and Biomedical Laboratories" ("BMBL"), 5th Ed., 2009.
- "CDC Guidelines for Safe Work Practices in Human and Animal Medical Diagnostic Laboratories," 2012.
- "Canadian Biosafety Standard" (CBS), 2nd Ed., 2015.
- "Canadian Biosafety Handbook" (CBH), 2nd Ed., 2015.

Many of these internationally accepted reference documents share the same basic principles: (1) a classification system for the biological agents or biological materials in so-called risk groups, often divided into four classes going from 1 (low) to 4 (high); (2) the understanding that increasing occupational and environmental risks require more stringent containment measures to work with that material, which is translated in a requirement for both risk assessment and risk management that is tailored to the activities performed with the biological materials, and (3) the description of containment measures, either result-oriented or more prescriptive as true containment or biosafety levels (**Table 1**).

Some of these reference documents have also served as the foundation for the development of national biosafety and biosecurity legislation, regulations and policies, either by including and refining the concepts mentioned in these documents or including the compliance with these documents as a requirement in the legislation.

Examples of Country- or Region-Specific Legislation

Due to the multiple objectives envisaged by biosafety and biosecurity (*vide infra*), regulatory requirements are most often part of legislation that is focusing on topics such as Worker

TABLE 1 | Overview of principles shared among internationally accepted reference documents for biosafety in containment.

Topic	WHO LBM	35001	BMBL	NIH G	CDC G	CBS + CBH
Risk groups	X	-	X	X	(X)	X
Activity based risk management	X	X	X	X	X	X
Containment measures – prescriptive	X	-	X	X	X	X
Containment measures – result oriented	-	X	-	-	X	X

Legend: WHO LBM, WHO Laboratory Biosafety Manual; 35001, ISO 35001:2019 Biorisk management for laboratories and other related organizations; BMBL, Biosafety in Microbiological and Biomedical Laboratories 5th ed.; NIH G, NIH Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules; CDC G, CDC Guidelines for Safe Work Practices in Human and Animal Medical Diagnostic Laboratories; CBS, Canadian Biosafety Standard (CBS), 2nd Ed.; CBH, Canadian Biosafety Handbook (CBH), 2nd Ed.

Protection, Activities with Genetically Modified Organisms (GMO), Activities with Pathogens (human, animal, plant, quarantine), Waste or Biosecurity.

We have compiled the specific references to biosafety and biosecurity aspects in these themes for key countries in **Appendix 1** – Part A. These overviews were prepared for Australia, Brazil, Canada, the European Union, Singapore and the United States of America, and they reflect the regulatory status at the time of compilation (period July – Dec 2019) as examples of different approaches (Readers are advised to consult the local regulations to have access to the updated and most recent information).

OVERLAPS WITH OTHER REGULATORY FRAMEWORKS THAT HAVE PROVISIONS ON HANDLING BIOLOGICAL MATERIALS

Both on the international and the regional or local level, additional provisions for handling of biological materials are imbedded in diverse regulatory texts, several of which on first sight would not be immediately recognized as being relevant for biosafety and biosecurity in containment. Many of them are related to the topic of transboundary movement, traceability, transport and occupational hygiene, and their link to biosafety and biosecurity for contained use is explained here further for some concrete examples.

Cartagena Protocol

The "Cartagena Protocol on Biosafety to the Convention on Biological Diversity" (SCBD, 2000) describes in its Article 18 that "LMOs [Living Modified Organisms] that are subject to intentional transboundary movement within the scope of the Protocol are [to be] handled, packaged, and transported under conditions of safety, taking into consideration relevant international rules and standards", thus clearly referring to existing rules and requirements for maintaining containment during transport. Specifically for LMOs that are destined for contained use it is stipulated that they should be clearly identified as LMOs, a requirement which is common to many GMO specific regulations in different countries, and shipment documentation should provide instructions for the safe handling, storage, transport and use, thereby ensuring containment. In addition, by means of Article 15 "Risk Assessment" (including **Annex III**) and Article 16 "Risk Management", the Cartagena Protocol is aligned with the concepts described in different internationally accepted reference documents for biosafety in containment (see International Framework and Guidance Documents).

Nagoya Protocol

The "Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization to the Convention on Biological Diversity" (SCBD, 2011) states that when benefits (either monetary or non-monetary) are arising from the utilization of genetic resources (e.g., in research) as well as during subsequent commercialization, that these benefits "shall be shared in a fair and equitable way with the Party providing such resources that is the country of origin of such resources or a Party that has acquired the genetic resources in accordance with the Convention". Although in principle not related to biosafety, the Nagoya Protocol implies that full traceability on when and where a certain genetic resource (i.e., biological material, or in some case arguably even digital sequence information) was first accessed, as well as how it was subsequently used, is maintained. Clearly specifying the identity of biological material and ensuring traceability is also a key element of biorisk management. Typically, this traceability involves both biological traceability (from one generation to the next) as well as physical traceability (when shipped from one location to another) and recording requires the information to be updated in inventories, which are also a prerequisite to identify the hazards associated with an activity. In addition, appropriate inventories for regulated materials are often a legal requirement from a biosafety contained use perspective in certain countries or regions.

Plant and Animal Health

In the section on "Protecting Animal and Plant Health" the efforts from the International Plant Protection Convention (IPPC) and the World Organization for Animal Health (OIE) in safeguarding containment when handling plant and animal pathogens, respectively, were highlighted.

However, many of the standards developed by these two organizations deal with topics such as import and export as well as traceability. This is especially important in case of newly emerging infections with the potential of world-wide epidemics. Checking the sanitary status of plant materials and animals prior to import or export reduces the risk of spreading diseases, while the recording of movements is imperative to allow for a quick and targeted response in case it does go wrong.

Occupational Hygiene

Occupational Hygiene, as defined by the International Occupational Hygiene Association (IOHA, 2020) is "the discipline of anticipating, recognizing, evaluating, and controlling health hazards in the working environment with the objective of protecting worker health and well-being and safeguarding the community at large". Also known under the term of Industrial Hygiene, it is typically part of an Occupational Safety and Health program, where it focuses on chemical, physical and biological agents in the workplace possibly causing illness or discomfort, and aims to avoid health effects through risk assessment and management. Although occupational hygiene and biosafety go hand in hand in terms of both intended and unintended exposure to biological agents, there is a clear difference in scope, being the general workplace as a whole vs. specific activities with biological materials, respectively. A clear example in this respect in the prevention against Legionnaires disease (*Legionella*), which is a typical workplace biological exposure monitored and managed by occupational hygiene, and generally not in scope of biosafety.

Transport Regulations

The UN Model Regulations from the UN Economic and Social Council's Committee of Experts (UNECE) on the Transport of Dangerous Goods describe the recommendations for transport of dangerous goods to safeguard workers' health and safety, property, or environment protection during all modes of transport. These dangerous goods are divided in 9 classes, one of which is devoted to toxic and infectious substances (Class 6), while GMOs are classified as miscellaneous dangerous substances (Class 9). For each class of dangerous goods, the UN Model Regulations cover aspects such as general packing requirements, labeling, and transport documents. Although they are only recommendations, they serve as the basis for national and international transport regulations, and as such, contribute to worldwide harmonization in this field (UNECE, 2020). For infectious materials, triple packaging (consisting out of leakproof primary and secondary receptacles) is the rule, to ensure containment of the biological materials during transport and in the event of accidents or incidents. As such, when biological materials are brought outside of containment for transport, appropriate packaging ensures protection from unintentional exposure or accidental release.

Specific references to the national or regional legislation for the above-mentioned biosafety-related topics are given in **Appendix 1** – Part B (Readers are advised to consult the local regulations to have access to the updated and most recent information).

CONCLUSION

Although biosafety and biosecurity serve different objectives, they are often addressed together, especially in a contained use setting. This discipline has a long-standing history, predating GMO-focused biosafety approaches, and continues to evolve as new insights and new techniques become available. The risk assessment and management practices are embedded in a vast and robust framework of international, regional and national regulations and guidance dealing with handling, storage, containment measures, waste management, transport, packaging, and labeling of biological organisms under contained use, including GMOs, thereby ensuring the protection of human, animal, and plant health as well as the environment. Local (national, regional) legislation may be influenced by policy priorities, leading to significant differences in the administrative aspects of how biosafety is regulated, however, the main principles and practices are shared worldwide. And, as experience has shown, when new developments in biotechnology, microbiology, and synthetic biology emerge, the existing frameworks and practices can be applied and tailored when needed.

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DB and PR co-developed the concept of the manuscript. DB wrote the first draft of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2020.00650/full#supplementary-material

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A Framework for Effective Bt Maize IRM Programs: Incorporation of Lessons Learned From *Busseola fusca* Resistance Development

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Bt maize is genetically engineered to express insecticidal proteins from the bacterium *Bacillus thuringiensis*. Bt maize is used extensively by South African farmers to reduce yield losses caused by lepidopteran larvae. Starting in the 2004/2005 season, severe *Busseola fusca*-associated damage to Cry1Ab-expressing Bt maize was noted by South African farmers. The unsatisfactory pest control was eventually attributed to the development of insect resistance to the Cry1Ab protein in the Bt maize hybrids. An assessment of the historical events surrounding the development of resistance by *B. fusca* showed that there was room for improvement both in the insect resistance management (IRM) strategy selected and the implementation of the strategy. With the recent arrival of fall armyworm (*Spodoptera frugiperda*) in Africa, it is important to have IRM programs that are appropriate for all of the pests that constitute the maize lepidopteran pest complex. After the identification of shortcomings in the IRM programs implemented in South Africa, a framework is proposed for effective Bt maize IRM programs. The IRM framework integrates pre-marketing research, post-marketing monitoring, and two-level remedial action plans (RAPs). The core of the framework is a regulator-approved IRM strategy that is based on comprehensive pre-marketing research and serves to guide stakeholders during the post-marketing phase. The framework will assist technology developers and regulators, especially those with nascent regulatory systems, to select and implement IRM strategies that facilitate sustainable pest management.

Keywords: insect resistance management, *Bacillus thuringiensis*, Cry1Ab, MON810, *Busseola fusca*, refuge compliance

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INTRODUCTION

Sub-Saharan Africa faces serious food security risks because its demand for cereals is expected to increase >300% by 2050 (van Ittersum et al., 2016). Maize (*Zea mays* L.) is one of the most important food crops in sub-Saharan Africa, with more than 300 million Africans depending on maize as their main food source.

Abbreviations: AC, Advisory Committee; EC, Executive Council; GM, genetically modified; GMO, genetically modified organism; HDR, high dose/refuge; IR, insect resistance; IRM, insect resistance management; RAPs, remedial action plans.

One of the options for increasing maize yields is reducing losses caused by lepidopteran maize pests, such as the African maize stalk borer, *Busseola fusca* (Fuller) (Noctuidae), and the fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Noctuidae). The development and commercialization of maize hybrids that have been genetically engineered to produce *Bacillus thuringiensis* (Bt) insecticidal proteins provide a powerful tool for the control of lepidopteran maize pests. There are two key types of Bt insecticidal proteins: Cry and Vip (Chakroun et al., 2016). Cry proteins, which are produced during sporulation and form crystalline inclusions, are released when the cell wall disintegrates, whereas Vip proteins are produced and secreted during the vegetative stage of growth (Chakroun et al., 2016). Maize expressing one or more Bt insecticidal proteins is called Bt maize.

The development, testing, and cultivation of Bt maize require functional biosafety systems, with enacted laws and adopted regimes and regulations for assessing the risks and benefits associated with genetically modified organisms (GMOs). The African Biosafety Network of Expertise (ABNE) was established to enhance the capacity of African countries to build functional biosafety regulatory systems (ABNE, 2020). However, there are significant differences in the status of the biosafety systems in different African countries (**Figure 1**). South Africa has a well-established GMO regulatory system, and in 1997, it became the first African country to approve commercial cultivation of Bt maize.

In South Africa, GMOs, such as Bt maize, are regulated under the GMO Act and the GMO Amendment Act (Act 23 of 2006), with the Registrar (housed within the Department of Agriculture, Land Reform and Rural Development) responsible for administering the Act. An independent, scientific Advisory Committee (AC) reviews applications and provides recommendations to the Executive Council (EC), which is the decision-making body. The opinions and perspectives in this paper are based, in part, on the author's experiences as a member of the AC and EC, but should not be construed to be those of either the AC, EC or members of these committees.

First-generation Bt maize produces a single insecticidal protein, e.g., Cry1Ab in the case of transformation event MON810. MON810 was approved for commercial cultivation in South Africa in 1997, with resistance development in *B. fusca* noted in the 2004/2005 season. The fall armyworm developed resistance to most Bt maize hybrids just 3 years after release in Brazil (Fatoretto et al., 2017), suggesting that there is a high risk of this pest developing resistance to Bt maize also in Africa. When considering the distribution of *B. fusca* and *S. frugiperda* in Africa (**Figure 1**) and the fact that MON810 hybrids are being made available to African countries through the TELA Maize Project (AATF, 2020), it is highly likely that inappropriate or poorly implemented insect resistance management (IRM) programs will have significant adverse effects on the sustainable use of MON810 and other Bt maize in these African countries.

The author believes that the lessons learned from South Africa's experience with MON810 and *B. fusca* will be of value to technology developers, regulators, and policy-makers in other countries, especially those that are developing GMO regulatory systems or have nascent systems and that are considering approving or have just approved Bt maize. This perspective paper should be seen in this context.

THE SOUTH AFRICAN EXPERIENCE WITH FIRST-GENERATION Bt MAIZE

On the basis of studies published between 2002 and 2009, Brookes and Barfoot (2018) reported that the average yield gains in South Africa for genetically modified (GM) maize with an insect resistance (IR) trait was 11.1%. Bt maize expressing Cry1Ab was reported to provide effective control against *B. fusca* until the 2004/2005 season when severe damage to Bt maize was noted (Van Wyk et al., 2009). The reduced control (>10% damaged plants) in the 2004/2005 season was eventually attributed to the development of IR to the Cry1Ab protein in MON810 maize (Van Rensburg, 2007; Kruger et al., 2011). Although the resistance is to the Cry1Ab protein, the resistance is often simply referred to as resistance to MON810.

Based on assessments of the 2007/2008 and 2008/2009 seasons, Kruger et al. (2011) concluded that resistance to MON810 occurred in the Christiana area (North West Province) and the Vaalharts area (Northern Cape Province), areas that are approximately 50 km apart. Field-collected larvae from Vaalharts were reared, without apparent problems, for four generations on Bt maize plants (Kruger et al., 2011). By 2012, *B. fusca* populations with resistance to Cry1-Ab expressing maize occurred throughout the maize production region of South Africa (Kruger et al., 2012).

Field resistance is defined as a genetically based decrease in the susceptibility of a population to a toxin caused by field exposure to the toxin (Tabashnik, 1994). Tabashnik and Carrière (2017) classified *B. fusca* resistance in South Africa as practical resistance (field-evolved, >50% resistant individuals in a population, and reduced efficacy of Bt crop in the field). Mutations that confer resistance to Cry1Ab in Bt maize, including a dominant resistant trait, have been reported for *B. fusca* populations (Campagne et al., 2013, 2017).

Post-2015 data on resistance to MON810 in South Africa are not readily available, as by 2015 the registrant had almost completely phased out MON810 and replaced it with the pyramid event MON89034 (Cry1A.105 and Cry2Ab2).

FACTORS PLAYING A ROLE IN THE DEVELOPMENT OF RESISTANCE TO MON810

In this section, a few factors that are likely to have played a key role in MON810-resistance development by *B. fusca* are highlighted.

Bt maize IRM programs in South Africa are almost entirely dependent on the high dose/refuge (HDR) strategy, which requires planting a refuge area composed of non-Bt maize that is in close proximity to the Bt maize field. In South Africa, there has historically been limited active

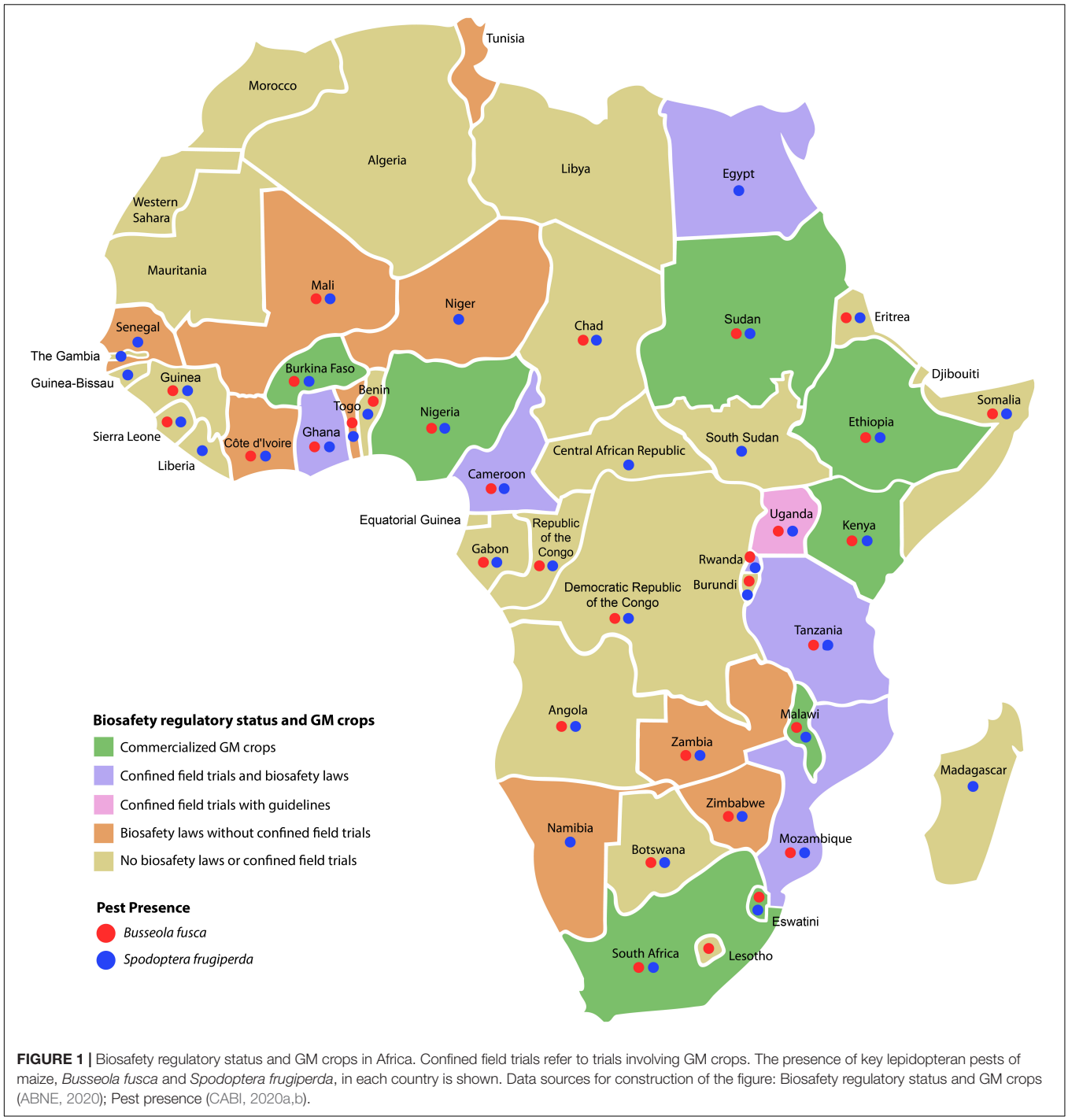

FIGURE 1 | Biosafety regulatory status and GM crops in Africa. Confined field trials refer to trials involving GM crops. The presence of key lepidopteran pests of maize, *Busseola fusca* and *Spodoptera frugiperda*, in each country is shown. Data sources for construction of the figure: Biosafety regulatory status and GM crops (ABNE, 2020); Pest presence (CABI, 2020a,b).

engagement between applicants and regulators around IRM strategy selection. In general, applicants present generic IRM plans, developed for pests not in South Africa, rather than pest- and event-specific plans that are fully interrogated for suitability. This is problematic, as the efficacy of the HDR strategy is dependent on it being suitable for the target insect and that all the assumptions of the HDR strategy have been met (Bourguet, 2004; USEPA, 2010; Gryspeirt and Grégoire, 2012).

A crucial requirement of the HDR strategy is that the Cry protein occurs in the maize at a high concentration, preferably 25 to 50 times the LD_{99} for the target pest (Caprio et al., 2000; USEPA, 2010). Prior to commercial cultivation, to the best of the author's knowledge, no data were available to show that the concentration of Cry1Ab in MON810 was several times the LD_{99} for South African populations of *B. fusca*.

A key principle underlying the HDR strategy is that homozygous resistant moths that may emerge from the Bt

maize are more likely to mate with one of a much bigger pool of susceptible moths that emerge from the refuge area, thus producing heterozygous resistant larvae that, if inheritance is functionally recessive, are expected to be killed by the Bt maize and slow the increase of the frequency of the Bt resistance allele (Gould, 1998; Bourguet, 2004). In this context, it is important to note that South African farmers are given two options for the conventional maize refuge size: 5% (which may not be treated with an insecticide) or 20% (which may be sprayed with an insecticide or non-Bt biopesticide). In South Africa, farmers almost never choose the 20% refuge option (Kruger et al., 2009, 2012). There is insufficient empirical evidence to determine if the 5% refuge size was adequate (i.e., produced enough susceptible adults) for *B. fusca* on MON810 in South Africa. Compliance with the requirement for planting a refuge is critical for the success of the HDR strategy. In 1998, one year after commercial release of MON810, only 7.7% of farmers that planted MON810 actually planted the refuge they were legally obligated to plant (Kruger et al., 2009).

A FRAMEWORK FOR DEVELOPMENT AND IMPLEMENTATION OF AN EFFECTIVE IRM PROGRAM

Based on the lessons learned from the South African experience with *B. fusca* and MON810, a framework for an effective Bt maize IRM program is proposed (**Figure 2**). This perspective paper does not aim to suggest a specific IRM strategy for *B. fusca* on MON810 in South Africa. Instead, the aim of the paper is to incorporate the lessons learned in a framework for developing and implementing an effective IRM program for any Bt crop–pest combination. The framework distils the overwhelming volume, especially for regulators and policymakers, of information on IRM down to a few critical steps. For readers seeking more information relating to the steps in the framework, the following references may be of use: Caprio et al. (2000), Glaser and Matten (2003), Matten et al. (2004), Head and Greenplate (2012), and Onstad (2013).

The IRM strategy development and selection phase is largely the responsibility of the applicant (usually the technology developer) that is seeking approval for commercial cultivation. There are four major parts in this selection phase (**Figure 2**).

The toxicity of the Bt proteins to geographically distinct populations of target pests should be determined in laboratory bioassays, using well-established bioassay methods (Siegfried et al., 2000, 2005). As Cry proteins produced by GM crops have properties that are different to naturally occurring Cry proteins or Cry proteins purified from GM bacteria (Latham et al., 2017), the choice of the Bt proteins used for these assessments needs to be carefully considered and justified by the applicant. Determination of the toxicity should include not only laboratory assessments, but assessments of the pest control provided by the Bt maize in field trials. Since the environment impacts on the expression levels of Bt proteins in Bt maize (Dutton et al., 2004; Trtikova et al., 2015), the efficacy field trials should be conducted under a range of agroclimatic conditions representative of the maize growing areas in the country. A key consideration is whether the target insects are able to complete their life cycles on the Bt maize, e.g., up to 2% of *B. fusca* larvae survived on MON810 hybrids in 1996/1997 field trials (Van Rensburg, 1999).

The expression levels of Bt proteins in Bt maize need to be determined (e.g., USEPA, 2010). To generate a complete view of the expression levels, the determinations need to be made under a range of growing conditions, ideally from the same field trials used to determine control efficacy. The expression level data generated under a range of environmental conditions will allow environment-related expression differences to be taken into consideration in the IRM strategy selection. The research should include assessment of the expression in different tissues at different plant growth stages, as expression levels in Bt maize hybrids can differ in different plant tissues at the same plant growth stage and also between the same plant tissue at different growth stages (SANBI, 2011).

The IRM strategy selection has to be based on empirical assessments of the toxicity and expression levels in the country for which commercial approval is being sought, and should take into account the population ecology of target pests (Head and Greenplate, 2012). The presence of any Cry-resistant pest populations in the country needs to be taken into consideration, as these populations may impact the efficacy of the strategy. For example, since cross-resistance among Cry1Ab and Cry1A.105 proteins is possible in lepidopteran maize pests (Hernández-Rodríguez et al., 2013), the strategy selection for MON89034 would need to consider the possibility that MON810-resistant populations are resistant to the Cry1A.105 protein in MON89034. The strategy selection cannot be based on a theoretical framework that is not supported by the in-country data. From the author's experience, IRM is often an afterthought in the overall risk assessment dossier and IRM strategies are frequently based on generic IRM strategies and data generated in other countries for different target pests. Mathematical modeling will facilitate integration of the data and selection of a scientifically sound IRM strategy (Mallet and Porter, 1992; Gryspeirt and Grégoire, 2012; Head and Greenplate, 2012).

The IRM strategy selection and IRM strategy dossier steps are separated in the framework to highlight the importance of selecting an appropriate IRM strategy, and because the IRM strategy dossier is part of the regulatory application process rather than a research process. The applicant should prepare a comprehensive IRM strategy dossier, which lays out the IRM strategy and justifies the suitability of the strategy. The dossier should include expression data, baseline susceptibility data for representative target pest populations, refuge requirements (including refuge size and location relative to Bt maize), and damage thresholds (i.e., levels of damage that are considered unacceptable). The foundation of the dossier should be comprehensive, multi-season, and multi-agroclimatic zone data generated during confined field trials in the country of application.

In the IRM strategy approval phase, the IRM strategy dossier should be reviewed by the regulator and, in consultation with the applicant, the strategy should be refined as required. The

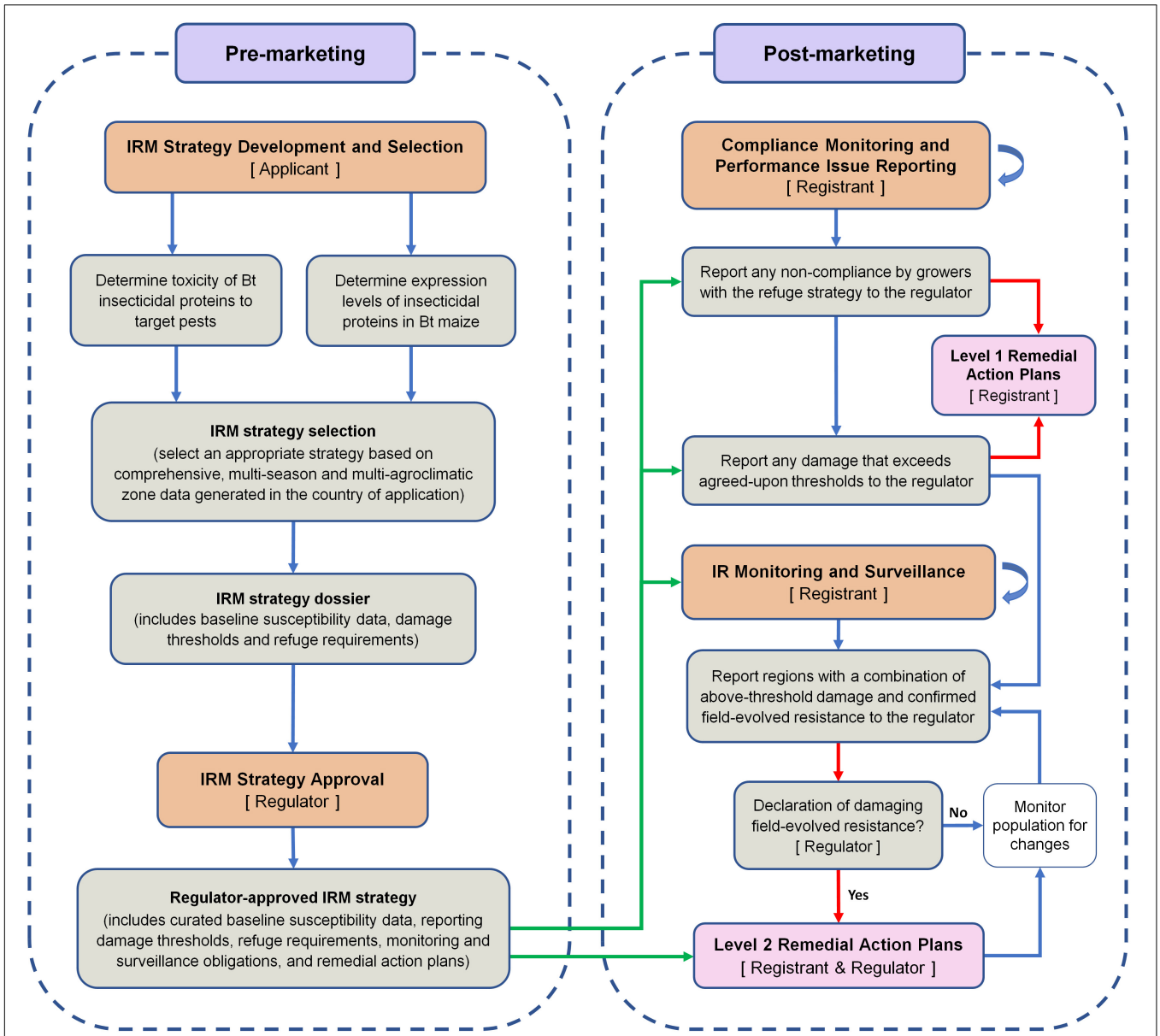

FIGURE 2 | A framework for the implementation of an effective pest- and event-specific Bt maize insect resistance management program. The stakeholders responsible for key steps are shown in square brackets. The core of the framework is the regulator-approved IRM strategy dossier. Arrows: green arrows show post-marketing activities that are guided by the regulator-approved IRM strategy; red arrows show the pathways that lead to the implementation of remedial action plans; blue curved arrows show that a process continues throughout the post-marketing phase. For the sake of simplicity, it is assumed that the applicant is also the technology developer/provider. Once approval has been granted for commercial cultivation, the applicant becomes the registrant.

key outcome of this phase is a regulator-approved IRM strategy. In this paper, the term regulator is used in a broad sense and may include officials from a number of government departments or agencies. In South Africa, the term regulator would refer primarily to the Office of the Registrar of the GMO Act, but also includes the EC (which consists of representatives of several government departments and the AC chairperson).

The regulator-approved IRM strategy is the core of the framework and guides the post-marketing monitoring, surveillance, and reporting. To aid in the post-marketing assessments, the approved IRM strategy should contain curated baseline susceptibility data, i.e., only baseline susceptibility data that were generated using well-established methods and are representative of the susceptibility of pest populations throughout the country of application should be included. In South Africa, baseline susceptibility data were apparently not generated, or at least not made readily available, prior to commercial cultivation of MON810. As a result, when unacceptable damage was first reported, there was disagreement as to whether the differences in control efficacy reflected

natural variation in the susceptibility of *B. fusca* populations to Cry1Ab. The absence of reliable baseline susceptibility data will permanently undermine post-marketing monitoring in IRM programs.

During the review of the IRM strategy, a comprehensive remedial action plan (RAP) should be agreed upon by the applicant and the regulator. The RAPs aim to contain and, if possible, eliminate resistant populations. The Biopesticide Registration Action Documents of the US Environmental Protection Agency contain examples of RAP actions (e.g., USEPA, 2010). The IRM framework proposed in this perspective paper introduces a two-level RAP approach, level 1 (L1) and level 2 (L2), with distinctively different triggers.

There are two triggers for L1 RAPs: non-compliance by farmers with the refuge strategy or on-farm damage exceeding the agreed-upon thresholds (**Figure 2**). Although non-compliance and threshold-exceeding damage are reported to the regulator, L1 RAPs are immediately implemented by the registrant. In South Africa, the registrant implemented several actions in response to above-threshold damage on MON810, including: (1) heightened communication and farmer training about the importance of IRM, (2) confirming that farmers are in possession of technology and stewardship agreements and reminding farmers of their IRM obligations under these agreements, (3) increased on-farm refuge compliance monitoring and attendance of mandatory training sessions of non-compliant farmers, and (4) spraying fields with >10% damage with insecticides. These steps were taken without confirmation of field-evolved resistance development, and the registrant communicated, as early as 2007, with the regulator about the alleged resistance. These L1-type actions may be considered successful, as full refuge compliance (i.e., planting a refuge of the correct size) improved markedly after 2007 and reached 75% in the 2013/2014 season (AfricaBio presentation, 2015). In the same season, partial compliance (refuge of incorrect size planted) was 17% and non-compliance was 8%. A further indication of the effectiveness of L1 actions was that from 2010 to 2014, farmer complaints as a percentage of total hectares of MON810 planted peaked at 2.5% in the 2012/2013 season and decreased to 1.8% ($\approx$ 49 000 ha) in the 2013/2014 season (AfricaBio presentation, 2015). During this period, the registrant and EC-initiated independent assessments kept the regulator up to date on the *B. fusca*-MON810 resistance issue.

A key part of an effective IRM program is monitoring and surveillance (Matten et al., 2004). IRM programs should ideally include pro-active monitoring, such as wide-scale application of diagnostic dose or discriminating dose assays and F_2 screens, which are useful for the detection of rare and recessive resistance alleles (Matten et al., 2004). In the case of *B. fusca* and MON810 in South Africa, the monitoring and surveillance program had significant scope for improvement and was primarily reactive. Performance issue reporting by farmers, who are legally obligated by technology agreements to report above-threshold damage to the registrant, appeared to serve as the primary surveillance tool. The framework presented in this paper does not include above-threshold damage under the monitoring and surveillance step, but instead uses it as a trigger for L1 RAPs and the need for thorough testing of insect populations from problem sites for the presence of field-evolved resistance. In the framework, a key step under monitoring and surveillance is the reporting of regions with a combination of above-threshold damage and confirmed field-evolved resistance to the regulator, especially if the resistance is spreading rapidly. The trigger for L2 RAPs is a declaration by the regulator of damaging, field-evolved resistance. The definition of what constitutes field-evolved resistance will need to be clearly stated in the IRM strategy dossier to avoid delays in implementing L2 RAPs. For the definition step, the paper of Tabashnik et al. (2014) may be useful. L2 RAPs may include, for example, cessation of sales in the affected and bordering areas, and extensive, area-wide insecticide applications. The L2 RAPs should be proportional to the scale of the problem and should aim to safeguard the technology and prevent the spread of resistant insect populations.

In the case of *B. fusca* and MON810, populations that were suspected of having developed resistance were, to the best of the author's knowledge, not assessed by the registrant for field-evolved resistance. However, external parties confirmed field-evolved resistance (Campagne et al., 2013). Early characterization of the resistance is important: e.g., when inheritance of resistance is non-recessive, as was the case for some *B. fusca* populations (Tabashnik and Carrière, 2017), the importance of insecticide application rather than relying on increased refuge compliance becomes apparent. Effective IRM programs should include assessments of field-evolved resistance and a clear pathway to L2 RAPs to avoid accelerated resistance evolution and rapid spread of resistance.

CONCLUSION

The framework presented in this paper will facilitate the development of case-specific Bt maize IRM programs that are effective for lepidopteran maize pests. The recent arrival of *S. frugiperda* in Africa means that effective Bt maize IRM programs are crucial for African countries, as two- and three-Cry protein Bt maize pyramids lost their ability to control this pest in Brazil within 3 years after their commercial release (Fatoretto et al., 2017). By clearly defining roles for stakeholders and pathways to RAPs, the IRM framework will assist in extending the useful life of Bt maize hybrids.

AUTHOR CONTRIBUTIONS

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

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Transportability of Conclusions From Confined Field Trials: A Case Study Using the Virus Resistant Transgenic Bean Developed in Brazil

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The conceptual framework for Data Transportability, builds on the premise that well-designed studies conducted for the environmental and food/feed risk assessment of transgenic crops may be transportable across geographies. Beyond individual data, provided that certain criteria are met, the general conclusions of comparative assessments of a transgenic crop with its conventional counterpart would also be transportable. In spite of this, many regulatory agencies still require in-country field trials to complete risk assessments of transgenic crops. A sub-team from ILSI Argentina's (International Life Sciences Institute, Argentina. www.ilsi.org.ar) Biotechnology Working Group tested the applicability of the transportability concept to the case of the *golden mosaic virus*-resistant transgenic bean, developed by EMBRAPA (EMBRAPA: Brazilian Agricultural Research Corporation). To this end, regulatory confined field trials (CFTs) carried out in Brazil to gather agro-phenotypic and compositional data were analyzed. The transportability of the conclusions of these studies to the bean cropping areas in Argentina was assessed as a conceptual exercise (with no intention to conclude on the biosafety of the common bean event). Comparative studies included the transgenic bean and its conventional parental line and were run in different agroecological environments so that any relevant differences could be observed. The main criteria to enable transportability were set by the sub-team and found to be met by the CFTs carried out in Brazil to inform a potential risk evaluation for Argentina.

Keywords: confined field trial, risk assessment, transportability of conclusions, transgenic bean, comparative studies, food/feed risk assessment, environmental risk assessment, criteria

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INTRODUCTION

Risk assessment for transgenic crops (also known as genetically modified crops) is typically conducted on a case-by-case basis using a weight-of-evidence approach. "Risk assessment based on an adequate problem formulation definition enables the development of plausible risk hypotheses that can be tested in order to identify and characterize risks" (Wolt et al., 2010).

Abbreviations: CFTs, Confined field trials; ERA, environmental risk assessment; BGMV, Bean Golden Mosaic Virus.

To assess risk, these hypotheses are tested using scientifically relevant information, which can derive from multiple sources, including field data. Results from well-designed studies conducted in laboratory, greenhouse, or in the field used for ERAs in one geography, are relevant to other geographies for the evaluation of the same or related transgenic crops (Garcia-Alonso et al., 2014; Ahmad et al., 2016). Furthermore, if these studies meet certain criteria, their conclusions should also be transportable. However, many regulatory agencies still require in-country field trials to complete risk assessments of transgenic crops intended for cultivation and even for import.

The concept of data transportability – data generated in one country used for the assessment in another country – focuses on the methodological quality of the studies and on the familiarity with crops, traits and receiving environments. As described by Capalbo et al., 2020 familiarity refers to the body of knowledge (evidence/data) and experience (of use, but also with risk assessment) with technologies and products that have undergone a risk assessment process or for which substantial data is available.

The Biotechnology Working Group from ILSI (International Life Science Institute) Argentina proposed to test the applicability of the concept of transportability to a real-world case. To this end, a sub-team was convened to investigate the transportability of conclusions from confined fields trials (CFTs) conducted in Brazil to Argentina, using as a case Embrapa 5.1, a transgenic common bean (*Phaseolus vulgaris*) resistant to the Bean Golden Mosaic Virus (BGMV) that was developed by EMBRAPA (Brazilian Agricultural Research Company).

The agro-phenotypic and compositional studies examined were based on a comparative field trial designed to measure biologically relevant differences between the transgenic crop and a conventional comparator for parameters that are informative for the risk assessment. Methodology and agronomic management of the studies, measured endpoints, and site selection, with focus on the diversity of tested environments were examined.

The exercise was limited to the applicability of the transportability concept, with no intention to conclude on the biosafety of the common bean event. This was a purely theoretical exercise as Embrapa 5.1 common bean is not under regulatory review in Argentina.

COMMON BEAN PRODUCTION IN BRAZIL AND ARGENTINA

Phaseolus vulgaris (common bean) is an annual species, native of Mesoamerica and South America, and its many varieties are grown worldwide for consumption (Alimentos Argentinos, 2016). Brazil is the main producer of common beans from the Mercosur region[1], being also the main consumer, as common bean varieties are a basic component of the Brazilian diet, with an average production of 3 million tons per year. In Brazil, common bean is widely cultivated throughout the territory (Schoonhoven and Voysest, 1994). Main production regions are Paraná, Minas Gerais, Bahía, Goiás, and Mato Grosso (Clemente et al., 2017; Silva, 2019; **Figure 1**).

Argentina follows Brazil with 473.389 tons, with 95% of the production cultivated in the northwestern region of the country, at altitudes ranging from 300 to 1,000 m (Schoonhoven and Voysest, 1994; Alimentos Argentinos, 2016; Informe de Cadenas de Valor, 2016; Calzada and Treboux, 2019; **Figure 1**).

The main diseases of the common bean affecting productivity, are caused by fungi, bacteria, and viruses, like the BGMV, *Bean dwarf mosaic virus*, *Bean common mosaic virus*, and *Cowpea mild mottle virus* (Morales and Jones, 2004; Vizgarra et al., 2016). The BGMV disease was described for the first time in Brazil in the 1960s (Morales and Jones, 2004). This viral disease is transmitted by the whitefly *Bemisia tabaci* from wild species or legumes such as soybeans, that act as reservoirs for the virus (Vizgarra et al., 2016). When soybean is harvested the whitefly is forced to find alternative hosts; this time overlaps with the planting season of the common bean, which is in its most susceptible stage to viral infections (Vizgarra et al., 2016). Expansion of the soybean cultivation area resulted in an increase of whitefly populations, leading to a rapid dissemination of BGMV in the main bean-producing states of Brazil (Costa, 1975; Morales, 1981; Gálvez and Morales, 1994), and also in Argentina, where the first detections were reported in 1983 in the North West region (Vizgarra et al., 2012).

As reported, there is no bean variety in South America with an adequate level of resistance to this virus (Morales and Jones, 2004). Historically the control of the BGMV has become dependent on cultural practices including chemical control of the vector, physical distance from soybean fields, and the use of varieties with some degree of tolerance to viral infections (Vizgarra et al., 2006, 2016).

Within this context, EMBRAPA embarked in a project to develop a transgenic common bean line resistant to BGMV. This highly resistant line was named Embrapa 5.1 and was designed using a gene silencing approach. The inserted construct triggers post transcriptional gene silencing, by degradation of the *rep* gene mRNA, which is involved in functions that are necessary for viral replication. By silencing *rep* expression, the viral replication is compromised upon infection, resulting in plants resistant to the virus (Bonfim et al., 2007; Faria and Arãgao, 2013).

Brazil's Technical National Commission on Biosafety (CTNBio) assessed the safety of Embrapa 5.1 for the environment and for human health, based on the studies submitted by EMBRAPA. As a result of this assessment, CTNBio approved Embrapa 5.1 for cultivation and consumption in 2011 (CTNBio, 2011).

All the information on Embrapa 5.1 reviewed during this exercise (section "Confined-Field Trials Conducted in Brazil for the Risk Assessment of Embrapa 5.1"), is publicly available at CTNBio's website, as part of the dossier submitted to the regulatory agency (CTNBio, 2011).

[1]Mercosur is the Southern common market comprising Argentina, Brazil, Paraguay and Uruguay; www.mercosur.int.

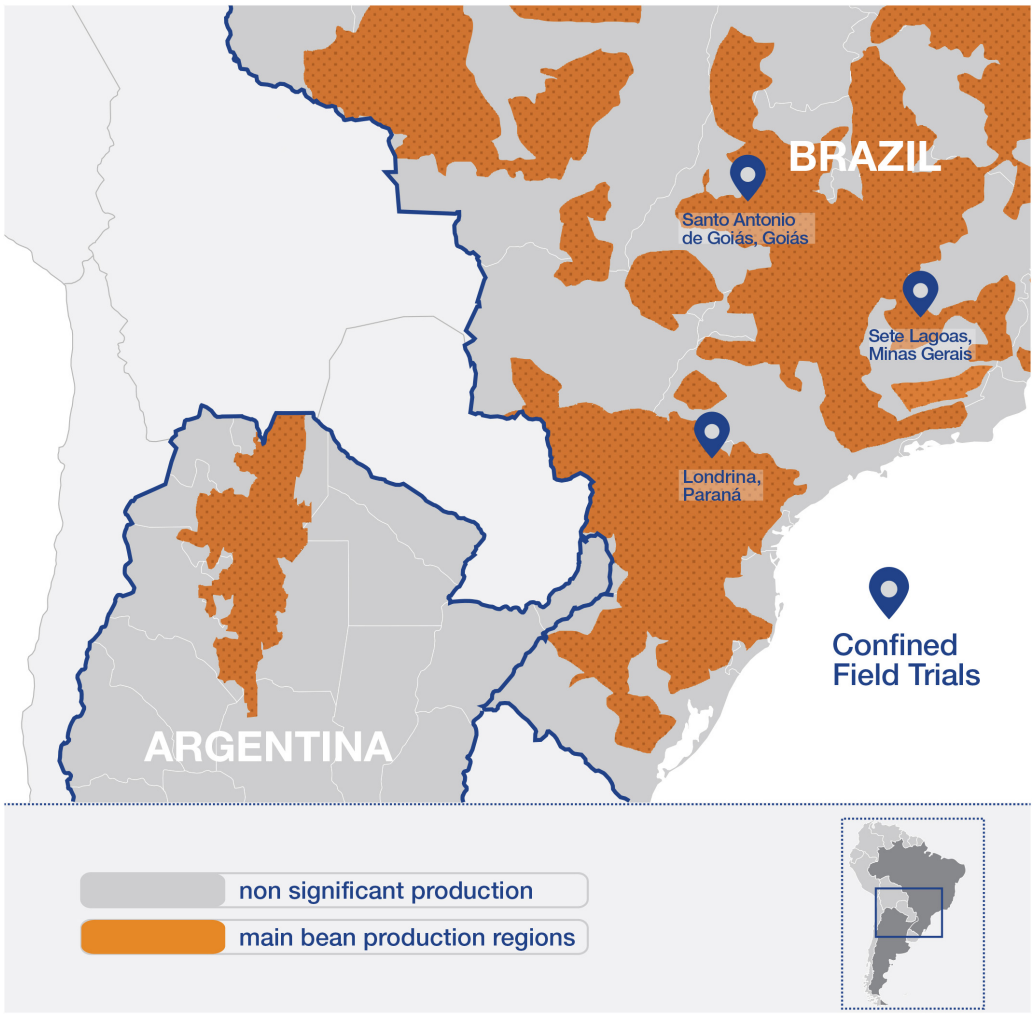

FIGURE 1 | Main bean production regions in Brazil and Argentina, and locations where CFTs were performed.

CONSIDERATIONS FOR TRANSPORTABILITY OF CONCLUSION

As Garcia-Alonso et al. (2014) described: "Field trials measurable endpoints vary, depending on the risk hypotheses being tested, but most of these studies are designed to identify differences between the transgenic crop and its non-transgenic counterpart, resulting from intended or unintended consequences of the genetic modification, across a range of agro-ecosystems."

From the environmental perspective, a key study to identify these differences at the phenotypical level, is the agro-phenotypic study. The measurable endpoints in the CFTs that inform the study are crop specific and generally encompass those characteristics relevant to plant emergence, vegetative growth and those related to the reproductive biology of the plant.

From the food and feed safety assessment perspective, the concept of substantial equivalence provides a basis to determine if the foods/feeds derived from a transgenic plant are as safe as the conventional counterparts (OECD, 1993; FAO/WHO, 1996; Codex Alimentarius, 2003). Typical endpoints in compositional studies are key nutrients, antinutrients, secondary metabolites, and toxins for the transgenic crop and its comparator (FAO/WHO, 1991; OECD, 1993; WHO, 1995; Jonas et al., 1996). The samples for these compositional analyses are obtained from edible plant parts harvested from CFTs. Several documents provide a reference framework for the compositional assessment, facilitating the harmonization and transportability of these studies; among these, CODEX guidelines are the international standard (Codex Alimentarius, 2003). Additionally, OECD consensus documents on composition of crops, containing key nutrients, anti-nutrients and toxicants are widely used to identify the relevant components for a specific crop in a comparative analysis (OECD[2]). Also, there are other crop composition databases that provide baseline data and ranges

[2]www.oecd.org/biotrack

of natural variability, established from multiple worldwide sources and seasons, for non-transgenic commercial cultivars (i.e., Agriculture & Food Systems Institute [AFSI], 2019).

As said, the comparative assessment between transgenic and non-transgenic crops involves plants grown side by side, that are therefore subject to the same environmental conditions and agronomic management. These are tested in different agro-climatic and agro-ecologic conditions within the crop production zones, under highly controlled conditions that will allow any biologically relevant differences arising from the gene insertion to be revealed.

CFT's that are run in different environments are suitable to inform the risk assessment, regardless of the country/regions where they have been conducted, as long as they cover a range of different environmental conditions. Only if a specific risk hypothesis is identified for a particular receiving environment, that cannot be addressed by the available information, local CFTs might be required to generate new information. There is published evidence supporting transportability of data generated in different geographies for the ERA of transgenic soybean and maize (Horak et al., 2015; Nakai et al., 2015; Ahmad et al., 2016; Heredia Díaz et al., 2017; Corrales Madrid et al., 2018; Clawson et al., 2019; Matsushita et al., 2020). These publications show that, even when climate and production practices may be different, the environmental safety conclusions from the comparative assessments are consistent across geographies provided that studies are run across a broad range of conditions. Therefore, replicating field studies in every country or region where the transgenic crop is intended to be released would add new data, but would not change the conclusions reached in previous studies from other geographies.

To assess the transportability of conclusions from studies based on CFTs, the following criteria were proposed:

- Appropriate experimental design and methodologies.
- Relevance and consistency of measured endpoints across studies.
- Diversity of environmental conditions in CFTs' locations within the crop production zones.

CONFINED-FIELD TRIALS CONDUCTED IN BRAZIL FOR THE RISK ASSESSMENT OF EMBRAPA 5.1

Based on the information submitted by the developer of Embrapa 5.1, the sub-team focused on the agro-phenotypic and compositional studies to evaluate the transportability of their conclusions, as these are the most typically reviewed studies in the environmental and food/feed risk assessment processes, respectively. The sites selected to perform these studies were representative of common bean cultivation areas in Brazil, in three distinct regions, designated as Santo Antonio de Goiás, Goiás (GO), Londrina, Paraná (PR) and Sete Lagoas, Minas Gerais (MG) in 2008 and 2009 (see **Figure 1**).

Assessment for Transportability of Conclusions

The sub-team applied the proposed criteria to assess the transportability of conclusions of these studies:

- Appropriate experimental design and methodologies:

Treatments included transgenic line Embrapa 5.1 and its conventional parent line (Olathe) as an appropriate comparator. Regarding the general agronomic management, cultural practices included: fertilization (after a soil analysis per site), irrigation, herbicide and insecticide applications. All these practices are typical for the bean production system. Likewise, the same crop management was uniformly applied across all plots at each site, helping to reduce the potential for non-trait related differences in pest pressure and agronomic performance among plots within a site. The study was conducted as a randomized complete block design, with 8 replicates per treatment at each location over 2 years. Data were analyzed by ANOVA using Statistical Analysis System (SAS) software to determine the effect of each treatment. Analyses were conducted across locations and years (location/year as a random factor) and for each location individually. Differences were considered significant at $P < 0.05$.

Regarding compositional studies, EMBRAPA developed a *de novo* common bean composition database, as a reference. To this end, eight common bean varieties were grown from 2003 to 2007 (5 years) in multiple locations. These reference materials provided a range of natural variation for each measured analyte. This database was later included in the OECD consensus document on compositional considerations for common bean (OECD, 2015).

- Relevance and consistency of the measured endpoints across studies:

The following characteristics were measured in the agro-phenotypic studies: yield, seedling emergence, seedling height, maximum width of the primary leaves, maximum length of the primary leaves, number of seeds per pod, weight of 100 seeds, pod length, pod width, seed length, seed width, thickness of seeds and flowering time. The sub-team considered that the selected parameters were appropriate and sufficient for risk characterization, as these adequately reflect the main phenotypic characteristics that are critical for productivity and common bean agronomic behavior.

In the compositional study, the endpoints considered for the analysis in raw and processed (cooked) beans included carbohydrates, vitamins B1 and B2, minerals, amino acids, and proximates. Anti-nutrients included phytic acid and trypsin inhibitors. These analytes are included in the recommendations of the OECD Consensus Document for common beans.

- Crop production areas where the CFTs were conducted:

As Faria and Arãgao (2013) mention, the edaphoclimatic conditions differed among locations. The sub-team reviewed the historical information on environmental factors (Instituto Nacional de Meteorología [INMET], 2020) for each location (GO, PR, and MG, **Figure 1**). Characteristics taken into

account to assess the diversity of environmental conditions, included site locations (latitude / longitude), historical water balance, and environmental factors: temperature, humidity, and precipitation. Although soil type is not a key parameter for data transportability (Garcia-Alonso et al., 2014), it was also taken into account as secondary element to discriminate environments. The CFTs' locations covered different bean production zones, and the evaluated characteristics taken together showed agronomically relevant differences between locations.

Based on this analysis of the three sets of criteria, the conclusions drawn from these studies were considered to be transportable for the purpose of a potential risk assessment in Argentina.

Summary of Results and Conclusions Described in the Agro-Phenotypic and Compositional Studies

There were no biologically relevant differences in the agro-phenotypic study comparing Embrapa 5.1 and its conventional parent line. The few statistically significant differences found for the measured endpoints were not consistent across locations or across years in a particular location. Thus, these differences were not associated with a specific location nor with the gene insertion and were considered random. The study reached to the conclusion that Embrapa 5.1 is agro-phenotypically equivalent to the conventional parent line.

Likewise, analysis of compositional results revealed no biologically relevant differences. The few statistically significant differences found for certain analytes were not consistent across locations or across years in a particular location. Thus, these differences were not associated with the gene insertion. Furthermore, all values fell within the range of conventional common bean varieties with a history of safe use in Brazil. The study concluded that Embrapa 5.1 was substantially equivalent to the conventional common bean in terms of composition and nutritional value.

DISCUSSION AND CONCLUSION

Based on the premise that "studies conducted in different countries may be relevant and can help risk assessors in making informed safety decisions" (Garcia-Alonso et al., 2014), recent reports support transportability showing that environmental safety conclusions from comparative assessments are consistent across geographies (Horak et al., 2015; Nakai et al., 2015; Ahmad et al., 2016; Heredia Díaz et al., 2017; Corrales Madrid et al., 2018; Clawson et al., 2019; Matsushita et al., 2020).

When a specific environmental risk hypothesis that is a particular concern for a receiving environment is identified during problem formulation, the need to consider the similarity of climatic conditions or agronomic practices could become relevant to enable transportability (Corrales Madrid et al., 2018). If after comparing environments those concerns are not addressed by the available studies, then local CFTs may be justified. Nevertheless, this would not impair transportability, as other studies will still provide data that add to the weight-of-evidence.

According to the proposed criteria for transportability of conclusions – the experimental design and methodologies, relevance and consistency of measured endpoints, and diversity of environmental conditions – the agro-phenotypic and compositional studies examined were considered appropriate for the conclusions to be transportable from Brazil to Argentina. Therefore, these conclusions could inform an eventual environmental and food / feed risk assessment of Embrapa 5.1 in Argentina.

The proposed criteria and the assessment methodology here presented, may help reduce unwarranted repeat of existing studies to conclude on environmental and/or food/feed safety of a transgenic crop. Transporting data and conclusions from CFTs can reduce the time and resources needed to conduct the risk assessment, reduce logistical and economic burdens on local, public sector and small private developers, and ultimately promote innovation by reducing unnecessary delays before beneficial technologies can be brought to market.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: http://ctnbio.mctic.gov.br/liberacao-comercial;jsessionid=6213F402341552C281162D71DD94A906.columba?p_p_id=110_INSTANCE_SqhWdohU4BvU&p_p_lifecycle=0&p_p_state=normal&p_p_mode=view&p_p_col_id=column-2&p_p_col_count=3&_110_INSTANCE_SqhWdohU4BvU_struts_action=%2Fdocument_library_display%2Fview_file_entry&_110_INSTANCE_SqhWdohU4BvU_redirect=http%3A%2F%2Fctnbio.mctic.gov.br%2Fliberacao-comercial%2F-%2Fdocument_library_display%2FSqhWdohU4BvU%2Fview%2F686135%3Bjsessionid%3D6213F402341552C281162D71DD94A906.columba&_110_INSTANCE_SqhWdohU4BvU_fileEntryId=686159#/liberacao-comercial/consultar-processo.

AUTHOR CONTRIBUTIONS

All authors participated in the drafting of this manuscript as individual experts in their fields, and the authors are solely responsible for the contents. Any views expressed in this manuscript are the views of the authors and do not necessarily represent the views of any organization, institution, or government with which they are affiliated or employed.

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Paraguay's Path Toward the Simplification of Procedures in the Approval of GE Crops

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Agricultural biotechnology was first regulated in Paraguay in 1997. The first update to the country's regulatory framework came in 2012, motivated by the need to keep up with current technologies. As part of this process, in late 2012, the Paraguayan Ministry of Agriculture (MAG) joined the Partnership for Biosafety Risk Assessment and Regulation, led by ILSI Research Foundation. The purpose of the program was the development of capacity building activities. As a result, the regulatory authorities in Paraguay incorporated the problem formulation approach to environmental risk assessment into their regulatory processes, leading to improved efficiency, with more timely decisions. Shifting to a problem formulation-based decision-making system was not straightforward, since practice and experience are always required to make professional risk assessors. Despite the continuity of approvals, there was a lag in the response time reflected in the number of events approved. During 2019, a simplified approval procedure for events that have been assessed by sound and experienced regulatory systems was introduced. Acceptance of third-country assessments can allow regulatory systems to make better use of their human, financial, and institutional resources, and stimulate inter-agency cooperation. In this work we aim to present the recent evolution of the regulatory system in Paraguay toward the establishment of a simplified procedure for GE crops that have been already assessed by sound and experienced regulatory systems, taking into account several scientific criteria. Concepts such as the familiarity, history of safe use, substantial equivalence, transportability, problem formulation, and the use of the consensus documents, developed by Organization for Economic Co-operation and Development (OECD), Food and Agriculture Organization of the United Nations (FAO), World Health Organization (WHO) and other institutions, favors the acceptance of decision documents issued by third countries. This requires the commitment of governments to support the stability of the institutions responsible for the regulatory implementation and also encourages countries to put work into the preparation and publication of decision documents, which are the basis for the commercialization of GE events.

Keywords: GE crops, regulatory system, acceptance of third-country assessments, simplified procedure, problem formulation

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INTRODUCTION

Biosafety regulations around the world have evolved on a "piece by piece" basis, frequently in response to demands or needs of the moment (McLean et al., 2002). Consequently, the different levels of institutional development, and in particular of the innovative and educational systems, the different trade positions and the perception of societies about biotechnology, led to national strategies for the construction of regulatory systems, which, with some exceptions, were individual, without international coordination mechanisms (Vicién and Alvarez, 2013).

Furthermore, biosafety regulatory systems deal with evolving scientific knowledge and technologies, and thus inherently require constant adjustment of their procedures and requirements (Vicién and Trigo, 2017).

In that context, for several decades, international organizations like the Food and Agriculture Organization of the United Nations (FAO), the World Health Organization (WHO), and the Organization for Economic Co-operation and Development (OECD) have worked on the development of assessment criteria for food and feed derived from GE crops. The Codex Alimentarius Commission has established guidelines with the assessment criteria to be considered, which most countries follow. From the analysis of regulatory frameworks of different countries, considerable similarities were found in the type of information required: expression of new substances, analysis of allergenic or toxic potential, compositional analysis, impacts on the nutritional profile, among others; however, there are still differences regarding required data and methodologies. This heterogeneity, which is not always science-based, contributes to the complexity of the risk assessment process, thus making it longer and increasing costs (Fernández Ríos et al., 2019).

Concerning environmental risk assessment, the data collected in confined field trials consist of agro-phenotypic characteristics, which are used mainly to assess unintended effects (Ladics et al., 2015), and to confirm that there are no changes in reproductive biology or growth habits that could have an adverse environmental impact (Nakai et al., 2015). These data are compared with one or more comparators grown in the same trial as the transgenic plant, and the comparator is usually the untransformed or near-isogenic parental line (Clawson et al., 2019). In most cases, transgenic plants are evaluated in multi-location confined field trials in the country of origin over multiple growing seasons, and there may be no scientific rationale for conducting additional trials. If there is, then the risk hypotheses should be clearly articulated. Nevertheless, still, many countries routinely require in-country confined field trials, even when data from confined field trials in the country of origin are enough to prove environmental safety (Roberts, 2019). That being the case, it has to be remarked that even though not harmonized, regulatory requirements for environmental risk assessment are very similar between regulatory systems, as most of the concerns related to potential harms are consistently addressed across different countries (Center for Environmental Risk Assessment [CERA], 2012).

The agricultural sector is one of the economic pillars of Paraguay in its contribution to the GDP, with the main crops being soybean, cassava, maize, wheat, sugar cane, and cotton. It should also be noted that Paraguay is the world's fourth exporter of soybean (MAG, 2020). The use of GE crops is important for the agricultural development of the country, making adequate access to products derived from biotechnology and its safe and sustainable incorporation to domestic production a vital requirement.

In 2020, the area planted with crops was 4.67 million hectares and consisted of soybean (3.56 million hectares), maize (1.08 million hectares), and cotton (18,000 hectares) (MAG, 2020). Since 2004, a total of 38 events[1] were approved in Paraguay for food, feed, and cultivation use; including cotton, maize, and soybean events. According to ISAAA (2018), Paraguay is the sixth largest producer of GE crops. Almost 94% of the soybean, 36% of the maize, and 56% of the cotton planted in the country are GE.

Keeping that context in mind, in this work we aim to present the recent evolution of the regulatory system in Paraguay toward the establishment of a simplified procedure for GE crops that have been already assessed by transparent and experienced regulatory systems, taking into account several scientific criteria.

THE BEGINNINGS

Agricultural biotechnology was first regulated in Paraguay in 1997. In 2012, the system was adjusted through the creation of the National Agricultural and Forestry Biosafety Commission (CONBIO), "with the mission to manage, analyze, and issue recommendations on all matters related to the introduction, confined field trials, pre-commercial and commercial release, and other intended uses of GE crops" (MAG, 2012).

One feature of GE crop applications for commercial release in Paraguay is that the transformation events have been in the global market for a while, and have thus been submitted to the scrutiny of regulatory systems that are sound and with experience in risk-assessment. There have been no applications for materials that are in the process of being developed locally or in a counter station development in the Northern Hemisphere.

Risk analysis followed a check-list criterion with exhaustive forms that did not clearly distinguish the differences between risk evaluation and risk management, despite having extensive information on approvals in third countries. There was a lack of a methodological framework on which to base the risk hypotheses that were applicable in the country's conditions.

The first transgenic crop was approved in 2004; 40-3-2/GTS40-3-2 Roundup Ready soybean. From 2004 to 2012, seven GE events were approved (**Figure 1**).

[1]Reports on the amount of approved GE events may vary depending on whether the parental lines and intermediate combinations approved through a single legal instrument are counted. For this work, we used the Biotrack Product Database (OECD) entry on Paraguay along with Decision documents from the Paraguayan government.

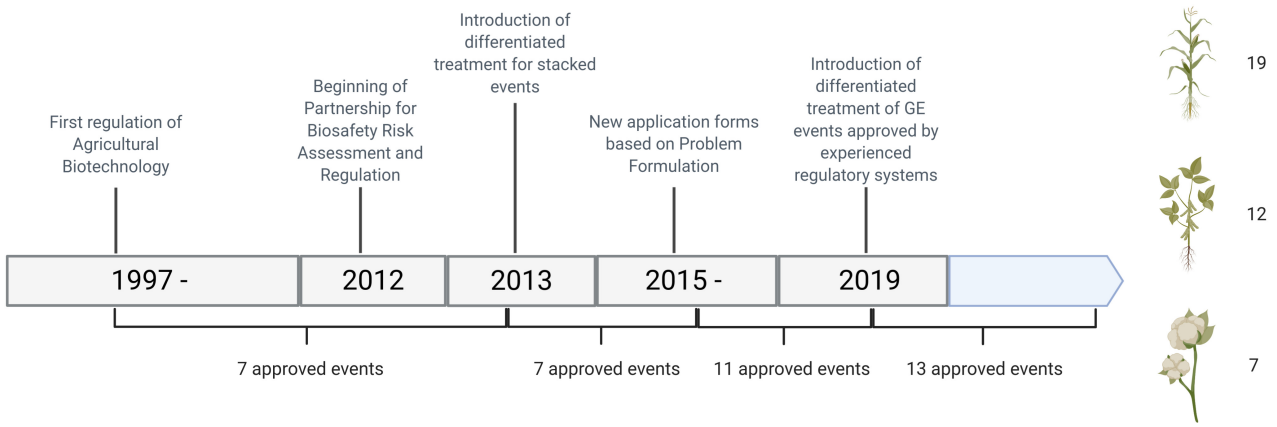

FIGURE 1 | Number of GE event approvals in Paraguay from 2004 to 2019, divided by stage of development of the regulatory system.

SOME LESSONS FROM A COLLABORATIVE PROGRAM

In late 2012, the Paraguayan Ministry of Agriculture (MAG) joined the Partnership for Biosafety Risk Assessment and Regulation, by means of the signature of a Memorandum of Understanding between the National Agricultural and Forestry Commission and the International Life Science Institute (ILSI) Research Foundation. With the aim of strengthening the technical capacity of stakeholders in developing countries regarding biosafety risk assessment and regulation, this collaborative program was framed within a global project led by ILSI Research Foundation and funded by the World Bank (McLean and Roberts, 2015).

Through this partnership, ILSI developed a capacity building program for Paraguay, based on feedback received from Paraguayan government representatives and stakeholders in agricultural biotechnology. Suggestions and recommendations from participants were also incorporated along with the implementation phase of the program (Fernández Ríos et al., 2018).

Regarding the activities, they "included building a knowledge base focused on developing effective skills on Problem Formulation for ERA of GE crops with a hands-on approach; analysis on key elements and procedures of a regulatory system for confined field trials for each stage in the development cycle of a GE crop; special considerations to the cases of non-target organisms and stacked event crops and safety assessment of foods derived from GE plants" (Soerensen et al., 2014). Seminars and workshops on agricultural biotechnology aimed at a wider, interested audience, and specific working sessions for regulators, scientists and graduate students directly involved in risk assessment activities, with in-depth discussions of risk assessment concepts and tools, using a hands-on methodology were organized (McLean and Roberts, 2015; Fernández Ríos et al., 2018).

A critical factor for the program's favorable outcome was the committed and coordinated effort of all participants from CONBIO, ILSI Research Foundation, and ILSI Argentina toward its implementation and the subsequent monitoring of its results. Other contributors were the National University of Asunción and the Argentine Council for Information and Development of Biotechnology (Argenbio), IICA's office in Paraguay (Inter-American Institute for Cooperation on Agriculture), and the Institute of Agricultural Biotechnology in Paraguay (INBIO) (Soerensen et al., 2014).

The national regulatory authorities in Paraguay incorporated the problem formulation approach to environmental risk assessment into their regulatory processes, leading to an improvement in the regulatory system, which could be shown by the implementation of more timely decisions on the use of new GE crop varieties for commercial release. In this regard, "the time for decision making by the national regulatory authority was reduced from 2 years to 3 months" (McLean and Roberts, 2015). Between June 2013 and February 2014, seven GE events were approved.

In addition to this, a Ministerial Resolution dictated the differentiated treatment for stacked events whose parental lines had already been approved (MAG, 2013).

The unifying conceptual tools for the environmental and food/feed problem formulation-based risk assessment of GE crops (Wolt et al., 2010; Garcia-Alonso, 2013) were crucial to provide a firm scientific foundation to decision-making. Upon completion of the capacity building program, this deeper understanding of the scientific ground underlying biosafety regulation led to the development of science-based risk assessment guidelines and application forms for confined field trials and for commercial release of GE crops (which includes both food/feed and environmental evaluations), based on the problem formulation methodology (Soerensen et al., 2014; MAG, 2015).

THE TRANSITION

The transition from the so-called "check-list" approach – applied from 1997 until 2012 in food/feed and environmental risk

assessments – to one based on problem formulation was not a minor task, as the learning curve of the regulators and the time needed to adjust are generally underestimated. Despite having new guidelines for evaluating applications, the main issue was the integration of the problem formulation process within the risk assessment into everyday work and a clear identification of protection goals (Garcia-Alonso and Raybould, 2014).

In this regard, since the capacity building program ended in 2015, the program partners have implemented follow-up periodical meetings with the participants with a hands-on methodology to discuss particular topics or share new information, developments and publications in order to keep up improving the regulatory system (Fernández Ríos et al., 2018).

In spite of the program's success in terms of capacity building and the follow-up implemented, CONBIO was still facing numerous difficulties. Its members are not fully dedicated, but due to the very nature of the composition of CONBIO they hold positions at institutions which they are designated to represent at CONBIO, and thus have other responsibilities derived from their positions at those institutions, lengthening assessment process. This fact showed the importance of having even a small group of dedicated risk assessors that could perform evaluations in a timely manner.

In addition, members were frequently replaced, and the advisors appointed by member institutions to be a part of CONBIO were experts in their respective fields, but quite rarely in risk assessment, which often generated debates about apprehensions that would not arise with a group specifically dedicated to and specialized in risk assessment (Fernández Ríos et al., 2018). It was difficult for these newly arrived members to adjust to analyzing information based on regulatory science criteria and examining dossiers as a source of data that responds to risk hypotheses. That leads to the consideration that practice and experience are always required to make professional risk assessors, and this is a lengthy process. These difficulties faced by CONBIO are rooted in its organizational structure, and thus would require organizational modifications or a simplification of operational procedures.

Between 2015 and 2018, eleven events were approved. Despite the continuity of approvals, the response time was lengthened, due largely to the issues indicated above.

A SIMPLIFIED APPROVAL PROCEDURE

In this context, in 2019 members of CONBIO considered and proposed the introduction of a simplified approval procedure for events that have been assessed by sound and experienced regulatory systems, thus maintaining the regular procedure for those GE crops that have not been previously assessed (MAG, 2015). The simplified procedure applies for commercial approvals hence including both food and feed and environmental evaluations. This implies the acceptance of scientific opinion by the regulatory authority in the country where the GE crop has been approved but only when several criteria have been taken into consideration in the risk assessment performed by those regulatory authorities.

Through MAG's Resolutions 1030 and 1071 there was stated a differentiated treatment for the commercial release of novel GE crops and for GE crops that have been approved in third countries, whose scientific, technical and safety characteristics are well-founded (MAG, 2019a,b). As has already been indicated, Paraguay usually receives submissions to assess events that have been in the market for a while and have thus been submitted to the evaluation of regulatory systems that are sound and experienced. In addition, those countries usually share Paraguay's protection goals.

Paraguayan Ministry of Agriculture's Resolutions authorize taking into consideration the decision documents from third countries with regard to both human and animal food safety in the cases where these evaluations have been based on Codex Alimentarius, such as the Guidelines for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants (Codex Alimentarius, 2003) and carried out in countries with time-tested regulatory systems and transparent procedures.

Concerning environmental safety, assessments are accepted for GE crops that besides having been authorized for commercial planting in countries with sound regulatory systems, include in the decision documents considerations as follows: that the GE crop under review has been studied under different environmental conditions, behaving in the same way as the conventional non-GE counterpart; that it will be managed in an agronomic manner similar to any GE or conventional hybrid/variety of the species; another aspect is that Paraguay is not center of origin of that crop, and finally two relevant characteristics are that there are no related weeds in Paraguay with which the GE crop could cross-breed and that the main target pests and the main non-target arthropod species present in Paraguay have been taken into account in the GE risk assessment carried out in those countries.

During 2019, in the period immediately following the adoption of the simplified procedure for events with commercial authorizations in third countries, thirteen events were approved; most of them with herbicide tolerance and/or Lepidoptera resistance, traits for which there is an extensive body of literature and experience with the safety of the novel proteins involved.

SOME FINDINGS

So far, all applications for regulatory approvals in Paraguay have been for transformation events that were already in the global market, having been scrutinized by sound and experienced regulatory systems. There have been no requests to evaluate locally developed events. Besides, decision documents from said countries, where regulatory criteria are specified, have always been an important basis for the decision making in risk analysis in Paraguay. In other words, there is a history of using information and data from existing risk analyses, and the GE crops in consideration have been cultivated in a range of receiving environments. That is why it was considered appropriate to develop a simplified procedure that could allow regulatory authorities in Paraguay to focus human, financial and institutional resources in a manner that is commensurate

with risk (Center for Environmental Risk Assessment [CERA], 2012). **Figure 1** shows the number of approvals per period of development since the establishment of the regulatory system in Paraguay.

Prior to capacity-building activities, Paraguay approved events that had been cleared for marketing by an average of approximately eight countries and mostly consisted of single events. In the following period, from 2013 to 2014, events that had been launched commercially on average by seven countries were authorized. Again, most approvals were of single (non-stacked) events. From 2015 to 2018, which is the period just after the adoption of the new forms for commercial approval with a problem formulation approach, the country began to authorize mostly stacked events. Within this period, the events approved by Paraguay had previously been approved for commercialization on average by five countries. Finally, in the period immediately after the adoption of the simplified procedure for events with commercial planting authorizations in third countries, the events approved by Paraguay had previously been commercialized on average by three countries, again with a majority of stacked events approved.

Finally, GE crops approved in Paraguay through the simplified procedure were presented with prior approvals from Brazil (11 events), Argentina (8 events), Japan (7 events), Canada (5 events), United States (3 events). These regulatory systems are experienced, perform science-based food/feed and environmental risk assessments aided by the problem formulation approach, use consensus documents produced by the OECD and the Codex Alimentarius Commission, and have transparent GE event approval procedures.

FINAL REMARKS

Acceptance of third-country assessments can allow regulatory systems to make better use of their human, financial, and institutional resources, and stimulate inter-agency cooperation. As a first step toward acceptance, countries must have a clear understanding of the scientific grounds for the establishment of acceptance criteria. In the case of food safety, these criteria are sufficiently harmonized, which would facilitate acceptance. As for environmental risk assessments, the framework given by problem formulation methodology when reviewing decision documents is a basis for a common ground. It is always of the most crucial importance to develop proper risk hypotheses and to rely on regulators with solid backgrounds on risk-assessment. In addition, it is important to note that these processes will depend on the level of trust between the actors of the regulatory process, on the implementation of validated methodologies, and on the assurance of the quality and integrity of regulatory data.

Finally, several aspects must be considered by authorities of regulatory systems in order to incorporate procedures for the acceptance of decision documents from third countries. So that said procedures are to be appropriate to the regulatory system's context, which means, will cause the least amount of disruption to the existing regulatory framework, will take into account the country's protection goals and will be accepted/trusted by the public. Concepts such as the familiarity, history of safe use, substantial equivalence, transportability, problem formulation, and the use of the consensus documents, developed amongst others by OECD, FAO, WHO and other institutions, in turn, favors the establishment of the acceptance system. Nevertheless, this requires the commitment of governments to support the stability of the institutions responsible for the regulatory implementation and is also relevant that governments make an effort to prepare and publish decision documents which are the basis for authorizing commercialization of the events.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. The data can be found here: https://conbio.mag.gov.py/.

AUTHOR CONTRIBUTIONS

All authors participated in the drafting of this manuscript as individual experts in their fields, and are solely responsible for the contents.

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