BIOSAFETY OF GENETICALLY MODIFIED ORGANISMS, VOLUME II

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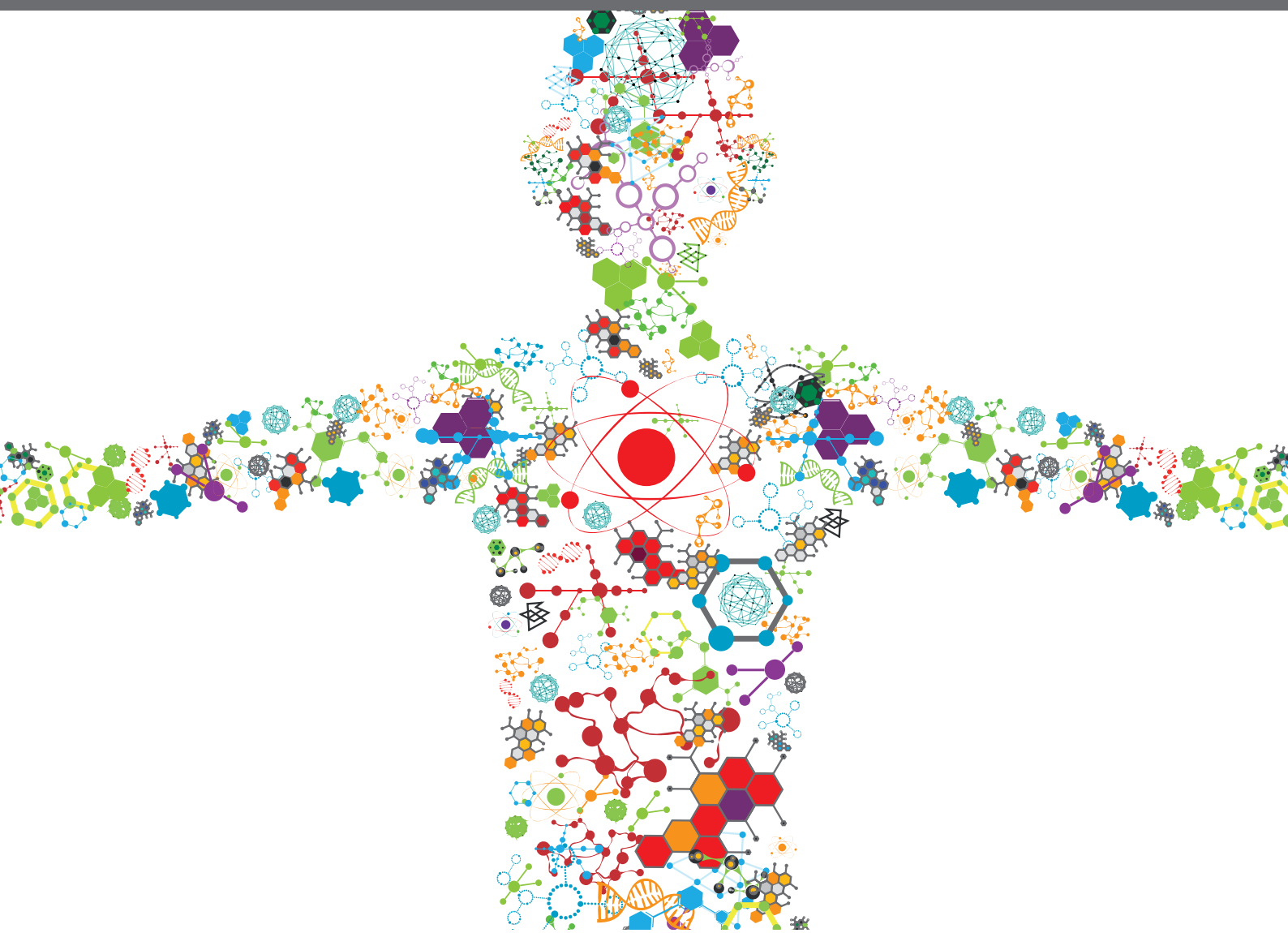

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BIOSAFETY OF GENETICALLY MODIFIED ORGANISMS, VOLUME II

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No Interactions of Stacked Bt Maize with the Non-target Aphid *Rhopalosiphum padi* and the Spider Mite *Tetranychus urticae*

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In the agroecosystem, genetically engineered plants producing insecticidal Cry proteins from *Bacillus thuringiensis* (Bt) interact with non-target herbivores and other elements of the food web. Stacked Bt crops expose herbivores to multiple Cry proteins simultaneously. In this study, the direct interactions between SmartStax® Bt maize producing six different Cry proteins and two herbivores with different feeding modes were investigated. Feeding on leaves of Bt maize had no effects on development time, fecundity, or longevity of the aphid *Rhopalosiphum padi* (Hemiptera: Aphididae), and no effects on the egg hatching time, development time, sex ratio, fecundity, and survival of the spider mite *Tetranychus urticae* (Acari: Tetranychidae). The results thus confirm the lack of effects on those species reported previously for some of the individual Cry proteins. In the Bt maize leaves, herbivore infestation did not result in a consistent change of Cry protein concentrations. However, occasional statistical differences between infested and non-infested leaves were observed for some Cry proteins and experimental repetitions. Overall, the study provides evidence that the Cry proteins in stacked Bt maize do not interact with two common non-target herbivores.

Keywords: Bt corn, Cry protein, SmartStax®, plant-insect-interactions, food web, arthropods, environmental risk assessment, non-target organism (NTO)

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INTRODUCTION

As an alternative to chemical insecticides, genetically engineered (GE) crops producing insecticidal Cry proteins from the bacterium *Bacillus thuringiensis* (Bt) have been developed. Cry proteins are known to have a relatively specific range of biological activity. Cry1 and Cry2 proteins exhibit activity for the larvae of some species of butterflies and moths (Lepidoptera) while Cry3 and Cry34Ab1/Cry35Ab1 proteins are active against larvae of some leaf beetles (Coleoptera: Chrysomelidae). To broaden the target spectrum, to delay the evolution of resistance in the target pest(s), and to simplify crop management, multiple Cry proteins have been combined into modern GE plants. SmartStax® maize produces the most combined GE traits of any currently commercially cultivated maize product, six different *cry* genes and two genes for herbicide tolerance (Head et al., 2017).

When insecticidal GE plants are grown in the field, the expressed Cry proteins may also interact with non-target herbivores and other components of the food web. Even though the Cry proteins engineered into Bt plants are known to be highly specific to Lepidoptera and Coleoptera, one concern with stacked Bt plants is that the different Cry proteins may interact synergistically and lead to unexpected non-target effects that are not observed when the individual Cry proteins are tested (Hilbeck and Otto, 2015). Potential interactions of different insecticidal proteins have been addressed in risk assessment studies using purified proteins and pest species that are sensitive to at least some of the used proteins. Graser et al. (2017) for example demonstrated that three different lepidopteran-active Cry proteins (Cry1Ab, Cry1F, Vip3A) acted in an additive way in tests with lepidopteran larvae. However, two coleopteran-active proteins (eCry3.1Ab, mCry3A) showed possible slight antagonism in tests with a coleopteran species. No effects of the coleopteran-active proteins were observed on the potency of the lepidopteran-active proteins against lepidopteran larvae and *vice versa*. This confirms earlier studies demonstrating no synergistic effects of the lepidopteran-active Cry1Ab and the coleopteran-active mCry3A (Raybould et al., 2012), of the lepidopteran-active Cry1Ac and Cry2Ab2 with another lepidopteran-active Bt protein, Vip3Aa19 (Levine et al., 2016), and of the coleopteran-active Cry3Bb1 with dsRNA used for an RNA-interference mode of action (Levine et al., 2015). While those studies used species that were sensitive to one of the insecticidal proteins and purified compounds, our study aimed at exploring potential effects of Bt maize producing several Cry proteins on non-target herbivores. We selected two non-target herbivores with different feeding modes: the aphid *Rhopalosiphum padi* (Hemiptera: Aphididae) and the spider mite *Tetranychus urticae* (Acari: Tetranychidae). Both species have a global distribution and are frequent pests of maize. While spider mites suck out mesophyll cells destructively, aphids feed on plant phloem. In consequence, spider mites ingest high amounts of Cry proteins, while aphids ingest only trace amounts, even when multiple Cry proteins are present (Svobodová et al., 2017). Previous laboratory studies with Bt maize producing single Cry1A proteins have shown no detrimental effects on the aphids *R. padi* (Dutton et al., 2002; Lumbierres et al., 2004), *Rhopalosiphum maidis* (Faria et al., 2008), and *Sitobion avenae* (Ramirez-Romero et al., 2008). Similarly, *T. urticae* was not affected when fed with maize producing Cry1Ab (Lozzia et al., 2000; Dutton et al., 2002), Cry1F (Guo et al., 2016), and Cry3Bb1 (Li and Romeis, 2010).

Based on the previously described results, the first hypothesis of the current study was that stacked Bt maize does not affect non-target herbivore performance compared to a genetically near non-Bt maize line. No data on potential effects of stacked Bt maize on spider mites and aphids are available and interaction studies of Cry proteins were done with sensitive target species in artificial diet systems using purified Cry proteins. Using SmartStax® maize, our study thus evaluated experimentally, if six different Cry proteins, some of which have not been tested on aphids and spider mites previously, may lead to unexpected interactions and effects on non-target species when produced in the plant context. Furthermore, *in planta* studies might also reveal potential indirect (plant-mediated) effects due to the transformation process and the plant's physiological responses (Ladics et al., 2015).

In addition to potential effects that Bt plants might have on non-target herbivores, the feeding of herbivores might also affect the plant. From the perspective of plant defense, herbivores could potentially influence the production of Cry proteins in the plant. Prager et al. (2014) claimed that the Cry1Ab and Cry3Bb1 concentrations in stacked Bt maize was reduced when plants were infected with spider mites. Such an effect could have important implications as it could lower the efficacy of the Bt plant against target herbivores. However, no such effects on Cry protein concentrations were observed with Bt cotton producing Cry1Ac and Cry2Ab and Bt maize producing Cry1F (Guo et al., 2016).

The second hypothesis of the current study thus was that herbivore infestation does not affect Cry protein concentrations in stacked Bt maize compared to non-infested Bt maize. To test this hypothesis, we worked with SmartStax® maize and two herbivores, *R. padi* and *T. urticae*. Using commercial ELISA kits, we measured five of the six plant-produced Cry proteins. This combination of Cry proteins and herbivores might reveal general patterns of herbivore effects on Cry protein expression if present.

MATERIALS AND METHODS

Maize Plants

Stacked Bt maize (Genuity® SmartStax®, event MON89034 × TC1507 × MON88017 × DAS-59122-7, Monsanto Company, St. Louis, MO, United States) and the nearest conventional non-Bt hybrid (EXP 258, Monsanto) were used for all experiments. SmartStax® expresses genes for the Lepidoptera-active Bt proteins Cry1A.105, Cry1F, and Cry2Ab, the Coleoptera-active proteins Cry3Bb1, Cry34Ab1, and Cry35Ab1, and two herbicide tolerance genes. Plants were grown individually in 12 L plastic pots and were fertilized with 40 g of slow release fertilizer (Manna, Wilhelm Haug GmbH, Ammerbuch, Germany) before sowing and weekly with 0.2–0.8 L of 0.2% liquid NPK fertilizer (Manna, Wilhelm Haug GmbH).

For the herbivore performance assays, Bt and non-Bt maize was grown in a climatic chamber (25 ± 1°C, 16:8 h L:D regime, 75 ± 5% humidity) and used when 9–10 leaves were expanded (approximately 4–5 weeks old plants). For the Cry-protein experiments, Bt maize was grown in a glasshouse (approximately 25°C) and used in the 10 leaves stage (5 weeks old).

Arthropods

Rhopalosiphum padi aphids and *T. urticae* spider mites were used from our own cultures on maize plants, which were started with individuals supplied by Syngenta Crop Protection Münchwilen AG (Stein, Switzerland). Both species were reared on Bt and non-Bt maize in the same climate chamber, but spatially separated to limit exchange between the two treatments.

Aphid Performance on Bt Maize

The experiment was conducted twice with 13 and 16 plants per maize treatment in the first and second run, respectively, resulting in a total of 29 plants per treatment. Bt and non-Bt plants were distributed alternately in blocks of 3–4 plants in the climate chamber. Two reproductive aphids from the culture were settled on the 5th, 6th, and 7th leaf and covered with one transparent plastic clip cage (3.5 cm diameter; 1 cm height) per leaf, resulting in a total of 39 and 48 clip cages per treatment in the first and second experimental run, respectively. Aphids for Bt maize or non-Bt maize were collected from the respective maize plants in the culture. Clip cages had a hole sealed with fine-mesh netting to provide air-circulation and foam rubber rings to gently seal against the leaf. In the first run of the experiment, all aphids except one neonate nymph were removed after 24 h (defined as day 0). Nymphs that were not sitting on the leaf were replaced by neonate nymphs within the following 2 days (unsuccessful settlement). The number of nymphs that needed to be replaced in this way was relatively high, but treatment-independent (20 in the Bt and 20 in the non-Bt treatment). In the second run of the experiment, the protocol was changed and 2–3 neonate nymphs were left on the leaves on day 0. After 24 h, the surplus nymphs were removed so that only one nymph remained in each clip cage. In this way, no nymphs needed to be replaced. Every day, aphid survival and the number of offspring produced by each aphid after reaching adulthood were recorded and neonate nymphs were removed. Aphids were monitored until death.

The nymphal development time was defined as the number of days from day 0 to the day when first offspring was observed. Adult longevity was calculated from the day when first nymphs were observed to the day the adult was found dead. Total fecundity was the sum of all offspring produced by an aphid during the experiment.

Spider Mite Performance on Bt Maize

The experiment was conducted twice with 15 plants per maize treatment in each run, resulting in a total of 30 plants per treatment. Bt and non-Bt plants were distributed alternately in blocks of 3–4 plants in the climate chamber. Spider mites were kept on leaf disks according to Li and Romeis (2010). Round leaf disks (ca. 2.5 cm diameter) were cut from the 5th, 6th, and 7th leaf of each plant and kept in a sandwich of two cotton pads. A hole (ca. 1 cm diameter) was cut in the middle of the upper pad. The cotton pads were wetted with tap water and kept in transparent plastic dishes (5 cm diameter, 1 cm height) covered with a ventilated lid. Using a binocular microscope, one female spider mite was transferred from the culture to each leaf disk using a fine paint brush with only 1 hair. Spider mites for Bt or non-Bt treatments were collected from the respective maize plants in the culture. The leaf disks were stored in a climate cabinet (MLR-352H-PE, Panasonic Biomedical, Etten-Leur, The Netherlands) at 25 ± 1°C, 16:8 h L:D regime, and 75 ± 5% relative humidity. Next day, the females were removed and all eggs except one were destroyed with a needle (defined as day 0). In the following, the cotton pads were rewetted, the hatching, survival, development, and reproduction of the spider mites were recorded, and the eggs were destroyed daily. Spider mites were transferred to new leaf disks every 3–4 days to ensure constant quality of food and exposure to the Cry proteins (Zurbrügg and Nentwig, 2009). New disks were cut next to the holes from the previous disks from the same leaves. The transfer of immobile larvae and nymphs (in the process of molting), was postponed to the next day. Once a male hatched from the deuteronymph, the experiment ended for this individual. In each cage with a newly hatched female, two (run 1) or one (run 2) male from the experiment or the culture was added to ensure mating. Only one male was used in the second run of the experiment, because mortality of males was very high in the first run, most likely because of competition among the two males. Males were removed after 3 days. Within those days, however, dead males were replaced. Egg fertility was examined in the second run of the experiment. When leaf disks were changed, the old leaf disks with eggs were incubated for 5–6 days and the number of unhatched eggs was counted and compared with the total number of eggs on the disk.

Egg hatching time was defined as the number of days from day 0 to the day when the larva hatched from the egg. Nymphal development time was the number of days from the day when the larva hatched to the day when the adult emerged. Female longevity was calculated from the day when the female emerged to the day it was found dead. Total fecundity included all eggs laid by a female during the experiment. Sex ratio was defined as the percentage of females.

Cry Protein Content in Maize after Aphid Infestation

Bt maize plants were grouped in six blocks of 3–4 plants in a large glasshouse cabin. The experiment was conducted twice with 20 and 22 plants in the first and second run, respectively. Before the plants were infested with aphids, four leaf disks (0.5 cm diameter) were punched from the middle part of the 9th leaf (counted from the base) using a common office hole-punch, placed in a 2 ml microtube and frozen at −80°C. The plants in half of the blocks (10 and 12 plants in the first and second run, respectively) were infested with aphids (approximately 100 aphids per plant), while the plants in the other half of the blocks remained non-infested. During the experiment, the non-infested plants were controlled daily and incidental aphids were removed. The experiment ended after 20 days, when infested plants were heavily populated with aphids. At the end of the experiment, four leaf disks (0.5 cm diameter) were sampled as described previously from the same leaf that had been sampled at the start of the experiment (9th leaf) and additionally from the first fully expanded leaf at the top (14th or 15th leaf).

Cry Protein Content in Maize after Spider Mite Infestation

Spider mites are much smaller than aphids, move through the air by ballooning, and it is practically impossible to control plants for unintended spider mite infestation. Therefore, infested and non-infested plants had to be kept in separate glasshouse

cabins. The experiment was conducted twice with 24 and 26 plants in the first and second run, respectively. The cabins were switched for the non-infested and infested treatments for the two runs. As described previously, four leaf disks were sampled from the 9th leaf before spider mite infestation. The plants in one cabin were infested with several 100 spider mites per plant, while the plants in the other cabin remained non-infested. The experiment ended after 20–22 days. Leaf disks were sampled once more from the 9th leaf and also from the first unfolded leaf at the top (14th or 15th leaf). At that time, leaves in the lower part of the plant were heavily damaged by spider mites, while the youngest leaves showed no (run 1) or little (run 2) damage. To document leaf damage, photos were taken with a Leica microscope/camera system (see Supplementary Figure S1). The climatic similarity of the two glasshouse cabins was confirmed with data loggers (Elpro Ecolog, Elpro-Buchs AG, Buchs, Switzerland), which revealed that the difference in mean temperature over the experimental period was smaller than 0.5°C and 15% RH.

Quantification of Cry Proteins

All collected leaf samples were lyophilized and weighed. Cry proteins were quantified individually with commercial double antibody sandwich (DAS) ELISA kits for Cry1Ab-1Ac (used to detect Cry1A.105), Cry1F, Cry2A, Cry3Bb1, and Cry34Ab1 (Agdia, Inc., Elkhart, IN, United States). The concentrations of the different Cry proteins were estimated as described by Svobodová et al. (2017) with the exception that standard curves were based on a single rectangular hyperbola model (SigmaPlot 13.0, Systat Software, Inc.). Leaf samples had to be diluted to achieve concentrations within the measurement range of the individual kits. If a large number of samples in one treatment was below the limit of detection or in the plateau area of the standard curve, all samples of the respective experiment were analyzed again for the respective Cry protein using a more appropriate dilution.

Data Analysis

All data used for statistical analysis, tables and figures for this publication can be found in the Supplementary Material online (**Supplementary Data Sheet 1**). Data from the herbivore performance assays were analyzed with linear or generalized linear models (GLMs) using R statistical software (R version 3.1.0, The R Foundation for Statistical Computing, Vienna, Austria). For all analyzed factors, contrasts were set to orthogonal. Applied models were full factorial for fixed factors.

Nymphal development time and adult longevity of both herbivore species were analyzed by generalized linear mixed-effects models (GLMMs) with Poisson distribution from the lme4 package. Fixed effects were treatment (Bt or non-Bt), leaf (5th, 6th, or 7th), and run (1, 2) and random effect was plant. Effects of factor and interactions were determined from an ANOVA table with Type III sum of squares (car package). Total fecundity of both species was analyzed similarly with linear mixed-effects models (LMM) from the lme4 package.

While the influence of the factors plant and leaf on spider mite egg fitness (which might affect hatching time) and sex were most likely minimal (predetermined by the female from the culture), run and treatment might have had an effect because spider mites were reared for several generations on Bt or non-Bt plants and the experiment was conducted at different points in time. Therefore, egg hatching time and sex ratio were analyzed by GLMs with the fixed factors treatment and run, a Poisson distribution for egg hatching time, and a binomial distribution (Logit link function) for sex ratio.

Total longevity of both species was analyzed among the Bt and non-Bt treatment with pooled data (no separation of other variables) (package survival). Censored data included all males of spider mites after emergence, and damaged and lost individuals of both species. The treatments were compared with a log-rank test in the survdiff function.

Power analyses were performed to determine the detectable differences (percentage difference of detectable treatment means relative to control means) based on the means and SEs of the non-Bt treatment (package pwr). For time and fecundity data, effect size d was calculated based on two-sided t-tests, the true sample size (N) of the non-Bt treatment, a power of 80%, alpha-level of 5%, and assuming equal sample sizes. Using d, a hypothetical second mean ($mean_{hypo}$) was calculated from the mean and the SD of the non-Bt group based on the equation (Cohen, 1988):

$$d = (mean_{non\text{-}Bt} - mean_{hypo})/SD_{non\text{-}Bt}$$

The detectable difference was then calculated from the proportions of both means:

$$det.diff = 1 - (mean_{hypo}/mean_{non\text{-}Bt})$$

For the sex ratio, effect size h was similarly calculated based on a test of two proportions. A hypothetical second proportion was calculated with h and the proportion of females in the non-Bt treatment ($p_{non\text{-}Bt}$) based on the equation (Cohen, 1988):

$$h = 2\arcsin(\sqrt{p1}) - 2\arcsin(\sqrt{p2})$$

The equation was once solved for p2 with $p1 = p_{non\text{-}Bt}$ and once for p1 with $p2 = p_{non\text{-}Bt}$. The detectable difference was then the difference between p1 and p2 and an average of both calculations (equation solved for p2 or p1) was reported.

Because ELISA estimates of Cry protein concentrations were highly variable and variances were inhomogeneous, they were analyzed visually based on 95% confidence intervals around the means. Significant differences between herbivore-infested and non-infested plants were concluded from non-overlapping confidence intervals.

RESULTS

Herbivore Performance Tests

Individual aphids developing in clip cages on leaves of Bt and non-Bt maize in the climate chamber had a similar nymphal development time, adult longevity, total fecundity, and total

TABLE 1 | Life table parameters of herbivores fed with SmartStax® maize (Bt) or the nearest conventional line (non-Bt).

Parameter	Mean ± SE (N)		*p*-value	Detectable difference (%)
	Bt	Non-Bt		
Rhopalosiphum padi				
Nymphal development time (d)	6.1 ± 0.14 (83)	5.9 ± 0.13 (86)	0.40	9
Adult longevity (d)	17.9 ± 0.80 (74)	17.9 ± 0.77 (75)	0.97	17
Total fecundity (no. of nymphs)	52.3 ± 2.65 (78)	54.0 ± 2.65 (84)	0.48	20
Total longevity (d)	24.1 ± 0.80 (89)	23.9 ± 0.85 (92)	0.85	–
Tetranychus urticae				
Egg hatching time (d)	4.0 ± 0.03 (89)	3.9 ± 0.04 (87)	0.82	4
Nymphal development time (d)	5.7 ± 0.09 (84)	5.8 ± 0.08 (80)	0.74	6
Sex ratio (% females)	60.7 ± 5.36 (84)	66.7 ± 5.27 (81)	0.35	20
Female longevity (d)	10.4 ± 0.81 (48)	10.1 ± 0.66 (51)	0.90	26
Total fecundity (no. of eggs)	81.3 ± 7.53 (48)	86.2 ± 7.30 (51)	0.78	34
Total longevity (d)	19.8 ± 0.79 (89)	19.4 ± 0.70 (87)	0.51	–

Results of statistical tests are provided (p-values). More details on statistics can be found in Supplementary Table S1. Power analyses were performed to determine the detectable differences based on proportions for sex ratio and t-tests for all other parameters, a power of 80%, an alpha-level of 5%, and the means and SDs of the non-Bt treatment assuming equal sample sizes and two-sided tests. N = number of replicates.

longevity (**Table 1**). Individual spider mites developing on leaf disks from Bt and non-Bt maize had a similar egg hatching time, nymphal development time, female longevity, sex ratio, total fecundity, and total longevity (**Table 1**). The experimental repetition (run) had a significant effect on all measured parameters in the aphid experiment and on total fecundity and female longevity in the spider mite experiment (Supplementary Table S1). The leaf where clip cages were positioned or from which leaf disks were cut (5th, 6th, or 7th) had no effect on any of the measured parameters (Supplementary Table S1).

The observed non-Bt means and standard deviations and the true sample sizes were used to calculate the detectable differences, i.e., the percentage difference of detectable treatment means relative to control means ($\alpha = 0.05$, power $= 0.8$). The lowest detectable differences (<10%) were observed for egg hatching time of spider mites and nymphal development time of aphids and spider mites (**Table 1**). Highest detectable differences of 20 and 34% were revealed for total fecundity of aphids and spider mites, respectively.

Egg fertility in the spider mite assay was high. Only 2.3 and 3.6% of the eggs in the non-Bt and Bt treatment, respectively, did not hatch.

Cry Protein Content after Herbivore Infestation

As anticipated, samples from the 9th leaf of Bt maize plants at time point 0 designated for herbivore infestation showed similar Cry protein concentrations to those designated to remain non-infested, based on overlapping 95% confidence intervals (**Figure 1** and Supplementary Table S2). One exception was Cry3Bb1 in the second run of the aphid experiment, where leaves of the non-infested treatment had significantly higher Cry3Bb1 concentrations than leaves from the designated infested plants.

After infestation with aphids, the 9th leaf showed a 40% higher concentration of Cry1F in the second run and a 50% lower concentration of Cry2Ab2 in the first run compared to non-infested leaves. The first fully expanded leaf at the top of aphid infested plants showed a 30% higher concentration of Cry34Ab1 in the first run compared to non-infested plants. After infestation with spider mites, the 9th leaf showed a higher concentration of Cry1A.105 and Cry1F in the first run and a higher concentration of Cry34Ab1 in both runs compared to non-infested leaves. The top leaf of spider mite infested plants showed a higher concentration of Cry3Bb1 and Cry34Ab1 in the first run compared to non-infested plants (**Figure 1** and Supplementary Table S2).

DISCUSSION

The performance assays with spider mites and aphids did not reveal any significant difference between the Bt and the non-Bt treatment for any of the measured life-table parameters. The power analysis (i.e., calculated detectable differences) provide information on the size of effects that could be detected with the given sample size and variation in the data, although those calculations, mainly based on simple *t*-tests, cannot be directly compared with the more complex statistical models used to determine differences between Bt and non-Bt treatments. The power analyses indicated that the smallest differences could be detected for nymphal development times (<10%), while parameters for adults (fecundity, longevity) were less sensitive (20–34%). This is not surprising because nymphal development was relatively uniform while adult survival and reproduction showed high variation. Similar detectable differences were reported for *T. urticae* feeding on maize by Li and Romeis (2010).

Aphids have been shown to ingest at most traces of Cry protein when feeding on Bt maize, as they feed on the phloem which does not contain high quantities of Cry proteins (Raps

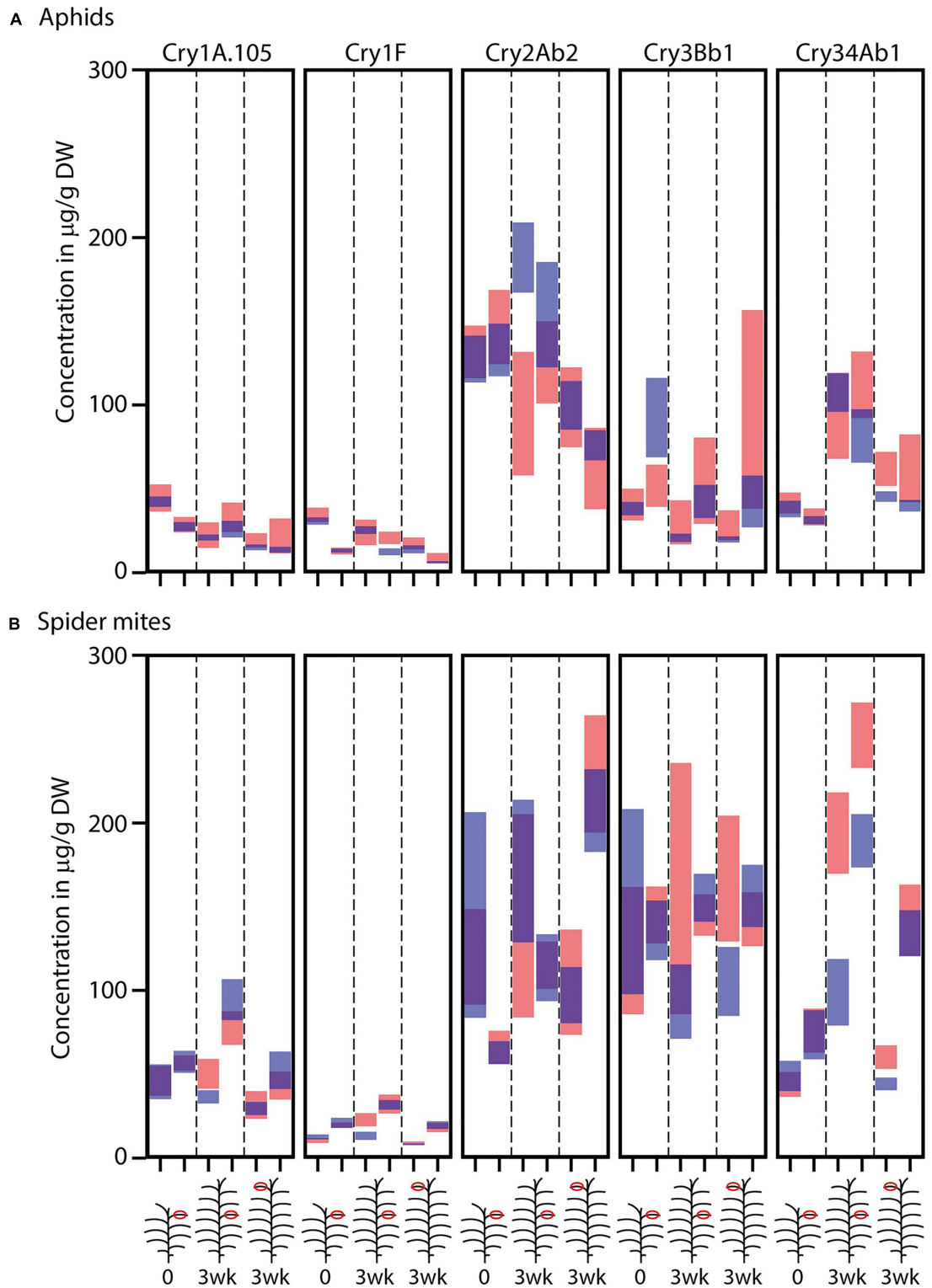

FIGURE 1 | Estimated Cry protein concentrations (µg/g dry weight) in leaves of SmartStax® maize before and after infestation with **(A)** aphids (*Rhopalosiphum padi*) or **(B)** spider mites (*Tetranychus urticae*). Samples taken from the 9th leaf counted from the bottom before infestation are labeled with 0, samples taken 3 weeks later are labeled with 3 wk. The maize illustrations indicate if samples were taken from the 9th leaf or from the 1st fully expanded leave at the top. Within each leaf/time category, the left and right bars represent the first and second run of the experiment, respectively. Each panel represents one Cry protein. Red bars indicate the 95% confidence intervals for the samples from the plants infested or foreseen to be infested with herbivores and blue bars indicate plants without herbivores. Overlapping confidence intervals have purple color. Detailed data can be found in Supplementary Table S2.

et al., 2001; Romeis and Meissle, 2011; Svobodová et al., 2017). In contrast, spider mites on SmartStax® were found to contain Cry proteins in concentrations of the same order of magnitude as leaves (Svobodová et al., 2017), as expected given mites consume leaf tissue which corresponds to plant level exposure.

Independent from the exposure to Cry proteins, unexpected differences due to the transformation process or differences among the Bt and non-Bt comparator not related to the produced Cry proteins could occur. Such effects, however, have not been observed in the current study. Our results confirm those of previous studies with Bt maize producing single Cry proteins. Li and Romeis (2010) reported no differences in life-table parameters for *T. urticae* feeding on Cry3Bb1-producing Bt maize compared to mites feeding on non-Bt maize. Similarly, *T. urticae* survival, development and reproduction were not affected when feeding on Cry1F-producing maize (Guo et al., 2016) or Cry1Ab-producing maize (Lozzia et al., 2000). Dutton et al. (2002) reported similar intrinsic rates of natural increase (r_m) for *T. urticae* and *R. padi* feeding on Cry1Ab-producing maize compared to non-Bt maize. Lumbierres et al. (2004) reported positive effects of Cry1Ab-producing Bt maize compared to the near isoline on the first generation of alate *R. padi* aphids, but opposite effects on the offspring of apterous mothers. The authors conclude that the observed differences in aphid development may be linked to changes in host-plant quality. Faria et al. (2008) did not observe differences in growth rate of individual *Rhopalosiphum maidis* aphids among six different pairs of Cry1Ab-producing Bt maize and their near-isolines. However, they observed more aphids on Bt maize in five of the six Bt/non-Bt maize pairs when large populations were left to develop for 5 weeks. Ramirez-Romero et al. (2008) reported no differences in developmental parameters of another aphid species, *Sitobion avenae*, when developing on Cry1Ab-producing Bt maize and its near-isoline.

One concern with stacked Bt crops is that the different Cry proteins may interact synergistically and result in adverse effects on the food web in a way not observed with plants producing only one Cry protein (Hilbeck and Otto, 2015). The present study, however, provides evidence that the different Cry proteins do not interact in a way that the two non-target herbivores are affected. Similarly, a companion study with the same plant material did not report any effects when Bt maize pollen and *T. urticae* and *R. padi* reared on Bt maize were fed to the predators *Chrysoperla carnea* (Neuroptera: Chrysopidae), *Phylloneta impressa* (Aranea: Theridiidae), and *Harmonia axyridis* (Coleoptera: Coccinellidae) (Svobodová et al., 2017). This supports the studies with sensitive herbivores that revealed no synergistic effects of different combinations of purified Cry proteins (Raybould et al., 2012; Levine et al., 2016; Graser et al., 2017) or Cry proteins with dsRNA (Levine et al., 2015).

Herbivore infestation had no consistent effect on Cry protein production by the plants. While in eight out of 40 comparisons, confidence intervals of Cry protein concentrations between infested and uninfested Bt maize plants did not overlap, these differences were only significant in one of two runs. One exception is Cry34Ab1 in the spider mite study, which was higher in the 9th leaf of infested plants in both runs (and in the top leaf in run 1). Higher Cry34Ab1 levels were also measured in top leaves of aphid infested plants, significant in the first run and almost in the second. While our results are in line with those reported by Guo et al. (2016) for Cry1F producing Bt maize infested with *T. urticae*, they are in contrast to Prager et al. (2014) who reported decreased levels of Cry1Ab and Cry3Bb1 when stacked Bt maize was infested with *Tetranychus cinnabarinus* spider mites at a comparable growth stage. No significant changes or elevated concentrations were evident with Cry1A.105 (although this protein might not be directly comparable to Cry1Ab) and Cry3Bb1 after infestation with spider mites in the present study.

The highly variable and sometimes very large confidence intervals demonstrate that ELISA estimates have to be interpreted with caution and should be regarded as semi-quantitative only. The commercial ELISA kits that we used are designed for qualitative detection of Cry proteins in leaves and our protocol for quantitative detection has not been truly validated. To ensure highest comparability among the treatments of one experimental run, we measured all samples from one Cry protein and one experimental run on the same plate. When several values of one Cry protein were out of range, the whole measurement (rather than individual samples) was repeated with a more appropriate dilution. Therefore, we are confident that the statistical comparisons that we made are based on comparable Cry protein concentration estimates. Repeated measurements for Cry1A.105 and Cry2Ab2 were generally in the same range as the first measurement, indicating that the additional freezing/thawing cycle did not degrade the Cry proteins to a large extent. In contrast, however, Cry3Bb1 showed ca. 80% lower values for the repeated samples and Cry1F showed 50% lower values. This clearly shows that values of the different Cry proteins and between experimental runs cannot be compared directly. Even more variation is likely when different protocols or different ELISA kit lots are used, thus comparing ELISA estimates across studies is cautioned (Nguyen et al., 2008).

Overall, the present laboratory and glasshouse studies provide evidence that the six Cry proteins (and two herbicide tolerance proteins) produced in SmartStax® maize did not interact with the two non-target herbivores studied, i.e., *R. padi* and *T. urticae*. Similar to Bt maize expressing single Cry proteins, stacked Bt maize is unlikely to affect populations of these herbivores via direct exposure. The hypotheses that stacked Bt maize does not affect non-target herbivore performance compared to a genetically near non-Bt maize line and that herbivore infestation does not affect Cry protein concentrations in stacked Bt maize compared to non-infested Bt maize are supported.

AUTHOR CONTRIBUTIONS

All authors contributed to the planning and designing of the experiments and to the drafting of the manuscript. YS and MM performed the bioassays and ELISA analyses. MM analyzed the data statistically.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00039/full#supplementary-material

DATA SHEET S1 | Raw data used for statistical analyses, tables and figures of this publication.

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ORIGINAL RESEARCH
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Toxicological and Biochemical Analyses Demonstrate the Absence of Lethal or Sublethal Effects of *cry1C*- or *cry2A*-Expressing *Bt* Rice on the Collembolan *Folsomia candida*

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Assessing the potential effects of insect-resistant genetically engineered (GE) plants on collembolans is important because these common soil arthropods may be exposed to insecticidal proteins produced in GE plants by ingestion of plant residues, crop pollen, or root exudates. Laboratory studies were conducted to evaluate the potential effects of two *Bacillus thuringiensis* (*Bt*)-rice lines expressing Cry1C and Cry2A in pollen and leaves and of their non-*Bt* conventional isolines on the fitness of the collembolan *Folsomia candida* and on the activities of its antioxidant-related enzymes, superoxide dismutase and peroxidase, and of its detoxification-related enzymes, glutathione reductase and glutathione *S*-transferase. Survival, development, reproduction, and the intrinsic rate of increase (r_m) were not significantly reduced when *F. candida* fed on the *Bt* rice pollen or leaf powder than on the non-*Bt* rice materials; these parameters, however, were significantly reduced when *F. candida* fed on non-*Bt* rice pollen or non-*Bt* leaf-based diets containing the protease inhibitor E-64 at 75 μg/g. The activities of the antioxidant-related and detoxification-related enzymes *in F. candida* were not significantly affected when *F. candida* fed on the *Bt* rice materials, but were significantly increased when *F. candida* fed on the non-*Bt* rice materials containing E-64. The results demonstrate that Cry1C and Cry2A are not toxic to *F. candida*, and also indicate the absence of unintended effects on the collembolan caused by any change in plant tissue nutritional composition due to foreign gene transformation.

Keywords: *Bt* rice pollen, *Bt* rice leaf, environmental risk assessment, ELISA, non-target effects, enzyme activity

INTRODUCTION

Multiple genetically engineered (GE) rice lines producing Cry1 and Cry2 proteins derived from *Bacillus thuringiensis* (*Bt* rice) have been developed in China and many of these lines can efficiently control target pests such as *Chilo suppressalis* and *Cnaphalocrocis medinalis* (both Lepidoptera: Crambidae) (Li et al., 2016). Planting of *Bt* rice cultivars thus has great potential for reducing

insecticide applications and to thereby benefit the environment and also human and animal health (Li et al., 2016; National Academies of Sciences Engineering Medicine [NASEM], 2016). Nevertheless, the cultivation of GE plants in general and of *Bt* rice, in particular, remains controversial because of safety concerns, one of which is the potential risk to valuable non-target organisms (Romeis et al., 2008; Li et al., 2017). Assessing the potential effects of *Bt* rice on non-target organisms is therefore an important part of the environmental risk assessment that must be conducted before the *Bt* rice can be commercially planted.

Recent studies have investigated the potential effects of *Bt* rice cultivars producing Cry1 and Cry2 proteins on non-target arthropods under laboratory or field conditions (reviewed by Li et al., 2016). Most of these studies have focused on plant-dwelling arthropods including predators belonging to different orders (Tian et al., 2010; Han et al., 2011, 2014, 2017; Li et al., 2014b, 2015; Wang et al., 2015, 2017; Meng et al., 2016), parasitic Hymenoptera (Han et al., 2015; Tian et al., 2017), and the silkworm *Bombyx mori* (Lepidoptera) (Yang et al., 2014). In all cases, these studies found that Cry proteins produced by *Bt* rice plants are very specific to target pest species in the order Lepidoptera and are not toxic to non-target species that do not belong to this order of insects (Li et al., 2016). Among the non-target species tested, only *B. mori* was adversely affected by consuming large amounts of *Bt* rice pollen containing Cry1C and Cry2A proteins (Yang et al., 2014). Sensitivity of *B. mori* to these proteins was expected because this species belongs to the target order Lepidoptera. The negative effects on *B. mori*, however, were only observed at exposure levels that far exceeded those expected under natural conditions, and the researchers, therefore, concluded that the planting of *Bt* rice would pose a negligible risk to *B. mori* (Yang et al., 2014).

Collembolans (springtails) are common arthropods in agricultural soils (Al-Deeb et al., 2003; Bai et al., 2010). In addition to being important consumers of plant residues and soil fungi, collembolans also help create humus (Bitzer et al., 2002). The common soil collembolan *Folsomia candida* (Collembola: Isotomidae) can be easily maintained in the laboratory and has been widely used as a standard test organism for assessing the non-target effects of insecticides and insecticidal GE plants (Fountain and Hopkin, 2005; Romeis et al., 2013; Yang et al., 2015; Zhang et al., 2017). This species has also been used in non-target risk assessment of *Bt* rice including rice lines expressing Cry1Ab or Cry1Ab/1Ac (Bai et al., 2010, 2011; Yuan et al., 2011, 2013, 2014). To our knowledge, information regarding the effects of *Bt* rice lines expressing the *cry1C* or *cry2A* genes on *F. candida* is limited to one study in which the collembolan was fed purified Cry1C and Cry2A proteins at concentrations that were 10 times higher than those in rice tissues. The results showed that *F. candida* was not sensitive to the Cry proteins (Yang et al., 2015). To date, the effects of feeding *F. candida* plant tissues from *Bt* rice lines containing Cry1C or Cry2A proteins (rather than purified proteins) have not been reported.

We therefore assessed the potential effects of ingestion of *Bt* rice pollen and leaves containing Cry1C or Cry2A proteins on *F. candida* with the hypothesis that consumption of these Bt rice material will not significantly affect the fitness of *F. candida.* Because plant tissues were used rather than purified toxins, we expected that the assessment would cover both the direct effects from the Cry proteins as well as possible indirect effects caused by unintended changes in plant composition as a consequence of the genetic transformation.

MATERIALS AND METHODS

Plant Material

The transgenic *Bt* rice cultivars T1C-19 and T2A-1 and their corresponding non-transformed near-isoline Minghui 63 (MH63) were used in the experiments. T1C-19 plants express a modified *cry1C* gene, and T2A-1 plants express a modified *cry2A* gene; the proteins encoded by both genes target lepidopteran rice pests. The non-*Bt* rice line MH63 is an elite indica restorer line for cytoplasmic male sterility in China.

The rice lines were simultaneously planted in three adjacent plots at the experimental field station of the Institute of Plant Protection, CAAS, near Langfang City, Hebei Province, China (39.5°N, 116.4°E). Each plot was approximately 0.1 ha, and the plots were separated by a 1-m ridge. The rice seeds were sown in a seeding bed on May 6, 2015. When the seedlings were at the four-leaf stage, they were transplanted in the field (June 14, 2015). The plants were cultivated according to local agricultural practices but without pesticide sprays.

A previous study showed that the Cry protein concentrations in the two transgenic rice lines are higher in the leaves than in the stems or roots and are highest in the leaves at the seedling stage (Wang et al., 2016). Rice leaves were therefore collected from >50 randomly selected seedlings before they were transplanted in the field on June 10, 2015. The collected leaves were immediately frozen in liquid nitrogen, lyophilized, ground to a fine powder, and stored at −20°C until they were used in the experiments.

When rice plants in the plots reached the flowering stage, rice pollen was collected daily from 3 to 13 September 2015 by shaking the rice tassels in a plastic bag. The collected pollen was air dried at room temperature for 48 h and subsequently passed through a fine mesh (0.125 mm) to remove anthers and contaminants. Pollen collected from each rice line was pooled and stored at −20°C until used in the experiments.

Test Insects

The *F. candida* specimens used in the current study were obtained from the same permanent laboratory colony as described in our previous studies (Yang et al., 2015; Zhang et al., 2017). The collembolans used in the experiments were 12 days old, which followed the OECD guidelines (Organisation for Economic Cooperation and Development [OECD], 2016) and which ensured that the specimens were mature (Snider, 1973). To obtain 12-day-old insects, we placed *F. candida* adults in Petri dishes with plaster in the bases and allowed the females to oviposit for 48 h before all adults were removed. The eggs hatched after approximately 7 days and the neonates that hatched on a single day were subsequently fed on the baker's yeast for 11 days and were then used in the experiments.

Rice Pollen Experiment

Our preliminary experiments showed that *F. candida* survival and development were similar on rice pollen (MH63) and on baker's yeast, which is a diet that is favored by the collembolan (*unpublished data*). This indicated that rice pollen is a suitable food for *F. candida* and can be used in dietary exposure experiments.

For the experiment, 12-day-old *F. candida* were randomly selected, individually placed in Petri dishes (diameter 35 mm; height 10 mm; with plaster in the base), and subjected to one of the following dietary treatments: (i) MH63 rice pollen (non-*Bt* rice pollen; negative control); (ii) T1C-19 rice pollen (*Bt* pollen containing Cry1C); (iii) T2A-1 rice pollen (*Bt* pollen containing Cry2A); and (iv) MH63 rice pollen mixed with E-64 [*trans*-epoxysuccinyl-L-leucylamido (4-guanidino) butane], which served as a positive control. The protease inhibitor E-64 was purchased from Sigma–Aldrich (St. Louis, MO, United States) and was used as a positive control because it is known to be toxic to *F. candida* (Yang et al., 2015). For preparation of the positive control, stock solutions of E-64 were diluted with distilled water to a defined concentration and then mixed with non-*Bt* rice pollen (75 μg/g pollen dry weight [DW]). To ensure that the control and pollen treatments were prepared similarly, the same volume of distilled water was mixed with *Bt* and non-*Bt* rice pollen. All of the prepared pollen diets were lyophilized and ground into powder 3 days before the initiation of the experiment and were stored at −20°C until used. Each treatment was represented by 50 replicates (one collembolan and dish per replicate). The diets were renewed every 2 days to prevent the degradation of the test compounds. Survival, the number of fecal pellets produced, and the numbers of eggs and offspring produced by each collembolan were recorded twice daily (9:00 a.m. and 9:00 p.m.). Every seven days, the surviving individuals were photographed with a photo-microscope, and body length and head width were measured using a scale in the microscope. The experiment, which was conducted in a climate chamber at 20 ± 1°C with 70 ± 5% RH and a 12-h light/12-h dark cycle, was terminated after 35 days.

To estimate the intrinsic rate of natural increase (r_m) of *F. candida*, individuals in each treatment were randomly assigned to one of three groups (16 or 17 individuals per group), resulting in three replicate groups per treatment. With the observed data, the r_m was calculated per group using the equation described in our previous study (Zhang et al., 2017).

Rice Leaf Experiment

Powder from leaves alone is known to be an unsuitable food for *F. candida* (Yu et al., 1997; Romeis et al., 2003). Our preliminary experiments revealed that rice leaf powder mixed with baker's yeast at a ratio of 10:1 (w: w) can support the survival, development, and reproduction of *F. candida* (*unpublished data*), and this mixture was used as the leaf-based diet in the current study.

The method used for the leaf-feeding experiment was similar to that used for the pollen-feeding experiment. The 12-day-old *F. candida* larvae were fed the following diets: (i) MH63 leaf powder mixed with baker's yeast (leaf-based diet; negative control); (ii) T1C-19 leaf-based diet; (iii) T2A-1 leaf-based diet; and (iv) MH63 leaf-based diet containing E-64. Each treatment was represented by 50 replicates (one individual and dish per replicate), and the same life table parameters including the r_m were recorded as described for the pollen-feeding experiment.

Uptake of Cry Protein by *F. candida* during the Feeding Experiments

To estimate the uptake of Cry1C or Cry2A protein by *F. candida* that fed on diets containing *Bt* rice pollen or leaf powder as described above, a separate assay was performed in which >30 Petri dishes (diameter 90 mm; height 10 mm), each containing >100 *F. candida* (12 days old) were provided with the *Bt* rice diets (pollen or leaf-based) or corresponding non-*Bt* diets as described above. After 7, 21, and 35 days of feeding, four samples per treatment (with 50–60 individuals per sample) were collected from different Petri dishes, resulting in a total of 72 samples (36 samples for pollen and 36 for leaves). The samples were frozen at −60°C for later ELISA analysis according to the methods described in our previous study (Li et al., 2015).

Stability and Bioactivity of Cry Proteins in the Diets

To evaluate the stability and bioactivity of Cry proteins in pollen or leaf-based diets during the feeding experiments, three 2- to 3-mg (FW) subsamples were collected from fresh diets that had been kept at −20° and from diets that had been exposed to *F. candida* for 2 days. The concentration and bioactivity of Cry proteins in the diets were analyzed by ELISA and sensitive-insect bioassays, respectively, according to the methods described in our previous study (Li et al., 2015).

Determination of Enzyme Activity

Folsomia candida (12 days old) were placed in Petri dishes (diameter 90 mm; height 10 mm; between 50 and 60 specimens per dish; >50 dishes in total) and exposed to non-*Bt* or *Bt* rice pollen or leaf-based diet or non-*Bt* rice pollen or leaf-based diet containing E-64 for 0, 7, 14, or 21 days as described before. At each sampling date, *F. candida* samples (200–300 individuals per sample, one sample per diet) were collected and stored at −20°C before the activities of the following enzymes were quantified in each sample: the antioxidant-related enzymes superoxide dismutase (SOD) and peroxidase (POD), and the detoxification-related enzymes glutathione (GR) and glutathione *S*-transferase (GST). The activities of these enzymes have been widely used as indicators of adverse effects caused by stomach poisons in *F. candida* and other arthropods (Bai et al., 2011; Yuan et al., 2011; Yang et al., 2015; Zhang et al., 2017). All enzyme activities were measured with enzyme kits from Nanjing Jiancheng Ltd., Co. (Nanjing, China) as described in our previous study (Zhang et al., 2017).

Data Analysis

Dunnett's tests were used to analyze the difference between the treatments and the negative control for the following parameters: body length, head width, number of fecal pellets, number of eggs,

and the intrinsic rate of increase. Hatching rates were analyzed by one-way ANOVA followed by HSD tests. Survival rates were analyzed with the Kaplan–Meier procedure and Logrank test. Cry protein concentrations and enzyme activities in *F. candida* collected on different days during the feeding assay were analyzed by repeated measures (RM-) ANOVA. In addition, Student's *t*-tests were used to compare Cry protein concentrations in the fresh pollen/leaf diets vs. pollen/leaf diets exposed to *F. candida* for 2 days. Chi-square tests were used to compare the mortalities of the *C. suppressalis* larvae in the sensitive-insect bioassay. All statistical analyses were conducted using the software package SPSS (version 15.0 for Windows, 2006). Unless noted otherwise, values are presented as means ± SE.

RESULTS

No Effects on Fitness of *F. candida* by Feeding on *Bt* Rice Pollen

The survival rates were >90% when *F. candida* fed on either *Bt* rice pollen (T1C-19 or T2A-1) or non-*Bt* rice pollen for 35 days, and there was no significant difference between any *Bt* pollen treatment and the control pollen treatment ($\chi^2 < 0.01$, $P = 0.99$ for T1C-19; $\chi^2 = 0.12$, $P = 0.73$ for T2A-1) (**Figure 1A**). However, the survival rate was significantly reduced when *F. candida* fed on non-*Bt* pollen containing E-64 ($\chi^2 = 17.660$, $P < 0.001$). Similarly, the mean body length and head width of *F. candida* were not affected by ingestion of *Bt* rice pollen ($P > 0.10$ for all sampling dates) (**Table 1**). In addition, the number of eggs produced per individual and the number of fecal pellets produced per individual were not affected by feeding on *Bt* rice pollen (**Figure 2**) (Dunnett's tests; T1C-19 pollen: $P = 0.27$ for number of eggs, and $P = 1.00$ for a number of fecal pellets; T2A-1 pollen: $P = 1.00$ for number of eggs, and $P = 0.93$ for a number of fecal pellets). All of these parameters, however, were significantly reduced when *F. candida* fed on the non-*Bt* rice pollen containing E-64 (Dunnett's tests, all $P < 0.01$). Interestingly, the egg hatching rate of *F. candida* did not significantly differ among the diets (one-way ANOVA; $F_{3,128} = 1.11$, $P = 0.35$).

The r_m values were 0.14 ± 0.001, 0.14 ± 0.003, 0.14 ± 0.005, and 0.11 ± 0.003 when *F. candida* fed on the pollen from non-*Bt* plants, T1C-19, T2A-1, and non-*Bt* plants containing E-64, respectively. The mean r_m values did not significantly differ between *Bt* and non-*Bt* pollen treatments (Dunnett's tests; $P = 0.66$ for T1C-19 pollen, $P = 0.99$ for T2A-1 pollen), but the mean r_m value was significantly reduced for *F. candida* that fed on the non-*Bt* pollen containing E-64 ($P = 0.004$).

Concentrations of Cry Proteins in *F. candida* Feeding on *Bt* Rice Pollen

As indicated by ELISA measurements, *F. candida* that fed on the *Bt* rice pollen diets contained Cry proteins. The concentration of Cry1C detected in *F. candida* on days 7, 21, and 35 was 38.49 ± 0.23, 41.89 ± 0.76, and 42.54 ± 1.44 ng/g DW, respectively, and the concentration significantly increased over time (RM-ANOVA, $F_{1,3} = 2963.9$, $P < 0.001$). The concentration of Cry2A detected in *F. candida* on days 7, 21, and 35 was 115.12 ± 6.17, 125.10 ± 0.19, and 252.85 ± 1.74 ng/g DW, respectively, and the concentration significantly increased over time ($F_{1,3} = 79431.2$, $P < 0.001$). No Cry protein was detected in *F. candida* that fed on the non-*Bt* rice pollen.

Stability and Bioactivity of Cry Proteins in Pollen Diets

According to ELISA measurements, the concentration of Cry1C protein in the freshly prepared Cry1C rice pollen diet was 1.82 ± 0.02 μg/g of pollen, and the concentration significantly decreased to 1.16 ± 0.02 μg/g of pollen after a 2-day feeding exposure to *F. candida* (Student's *t*-test; $t = 29.7$, df = 6,

TABLE 1 | Body length and head width of *Folsomia candida* that fed on a diet consisting of non-*Bt* pollen/leaf (MH63, negative control), *Bt* pollen/leaf (T1C-19: Cry1C, and T2A-1: Cry2A), or non-*Bt* pollen/leaf plus E-64 protein (E-64, positive control) for 35 days.

Parameters	Day	Treatment							
		Pollen diet				Leaf diet			
		MH63	T1C-19	T2A-1	E-64	MH63	T1C-19	T2A-1	E-64
Body length (mm)	7	1.071 ± 0.009	1.067 ± 0.010	1.077 ± 0.009	1.047 ± 0.009*	1.111 ± 0.010	1.081 ± 0.012	1.083 ± 0.012	1.058 ± 0.013*
	14	1.214 ± 0.010	1.205 ± 0.013	1.226 ± 0.012	1.157 ± 0.014*	1.194 ± 0.010	1.197 ± 0.012	1.188 ± 0.012	1.122 ± 0.015*
	21	1.287 ± 0.011	1.288 ± 0.014	1.287 ± 0.013	1.191 ± 0.013*	1.252 ± 0.009	1.255 ± 0.010	1.278 ± 0.011	1.161 ± 0.015*
	28	1.346 ± 0.012	1.347 ± 0.014	1.346 ± 0.014	1.233 ± 0.017*	1.313 ± 0.009	1.223 ± 0.010	1.348 ± 0.011	1.166 ± 0.029*
	35	1.399 ± 0.014	1.408 ± 0.015	1.407 ± 0.016	1.292 ± 0.008*	1.385 ± 0.008	1.381 ± 0.010	1.406 ± 0.080	1.216 ± 0.019*
Head width (mm)	7	0.204 ± 0.002	0.204 ± 0.001	0.204 ± 0.001	0.203 ± 0.001*	0.218 ± 0.002	0.213 ± 0.002	0.213 ± 0.002	0.205 ± 0.021*
	14	0.226 ± 0.001	0.225 ± 0.002	0.228 ± 0.001	0.219 ± 0.001*	0.235 ± 0.002	0.235 ± 0.002	0.235 ± 0.002	0.219 ± 0.024*
	21	0.238 ± 0.001	0.235 ± 0.002	0.236 ± 0.001	0.224 ± 0.001*	0.242 ± 0.002	0.239 ± 0.003	0.243 ± 0.002	0.225 ± 0.023*
	28	0.246 ± 0.001	0.244 ± 0.002	0.243 ± 0.001	0.228 ± 0.002*	0.248 ± 0.002	0.250 ± 0.001	0.251 ± 0.002	0.231 ± 0.034*
	35	0.253 ± 0.001	0.252 ± 0.002	0.251 ± 0.002	0.235 ± 0.002*	0.257 ± 0.001	0.258 ± 0.001	0.259 ± 0.002	0.236 ± 0.032*

Rice leaf powder was mixed with baker's yeast (10:1). The feeding experiment began with 12-day-old, mature insects; in the table, Day refers to days after the experiment began. Values are means ± SE, n = 50. Means in a row followed by an asterisk differ significantly from the respective negative control, i.e., MH63 pollen or leaf-based diets (P > 0.05, Dunnett's test).

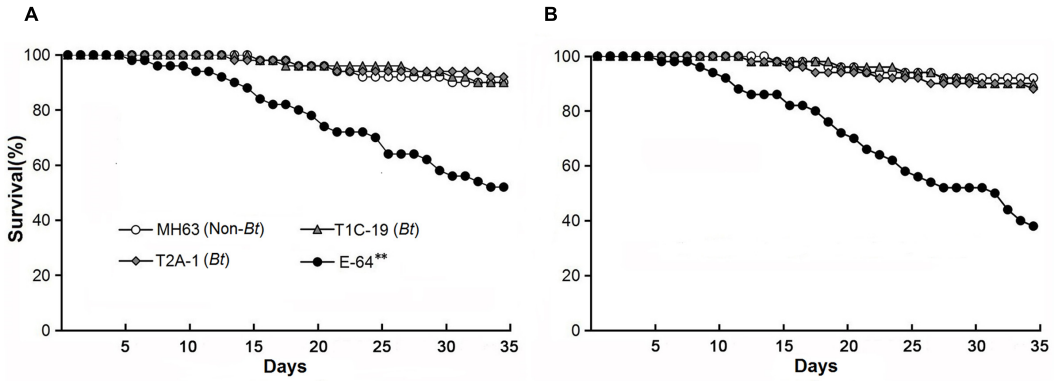

FIGURE 1 | Survival of *Folsomia candida* on a diet consisting of rice pollen **(A)** or a mixture of rice leaf powder and baker's yeast (10:1) **(B)**. The pollen and leaf power were obtained from either of two *Bt* rice lines (T1C-19: Cry1C, and T2A-1: Cry2A) or their non-*Bt* near isoline MH63 (negative control). For the positive control, the corresponding non-*Bt* rice tissue was supplemented with E-64. Asterisks indicate a significant difference between the treatment and the negative control (Logrank test, $P < 0.01$) ($n = 50$).

$P < 0.001$). The concentration of Cry2A detected in the freshly prepared Cry2A rice pollen diet was 26.41 ± 0.19 μg/g of pollen, and the concentration significantly decreased to 24.07 ± 0.12 μg/g of pollen after a 2-day feeding exposure to *F. candida* (Student's *t*-test; $t = 10.4$, df = 6, $P < 0.001$). No *Bt* protein was detected in non-*Bt* rice pollen.

The sensitive-insect bioassay showed that the mortality of *C. suppressalis* larvae was 3.3 ± 3.3% when the larvae were fed a diet containing the extract from non-*Bt* rice pollen for 7 days. The mortalities were 86.7 ± 3.3% or 83.3 ± 3.3% when *C. suppressalis* larvae were fed diets containing the extract from fresh Cry1C pollen (T1C-19) or Cry1C pollen that had been exposed to *F. candida* for 2 days. The mortalities were 80.0 ± 5.8% or 76.7 ± 3.3% when *C. suppressalis* larvae were fed diets containing the extract from fresh Cry2A pollen (T2A-1) or Cry2A pollen that had been exposed to *F. candida* for 2 days. Mortalities were not significantly different for larvae that fed on fresh diet vs. 2-day-old diet (Chi-square test; $U = 0.13$, df = 1, $P = 0.72$ for T1C-19 pollen; $U = 0.10$, df = 1, $P = 0.75$ for T2A-1 pollen).

No Effects on Fitness of *F. candida* by Feeding on *Bt* Rice Leaf Tissue

The survival rates were ≥88% when *F. candida* fed on the *Bt* rice (T1C-19 or T2A-1) leaf-based diets for 35 days, and there was no significant difference between any *Bt* leaf diet treatment and the control treatment ($\chi^2 = 0.11$, $P = 0.74$ for T1C-19; $\chi^2 = 0.53$, $P = 0.47$ for T2A-1) (**Figure 1B**). However, the survival rate was significantly reduced when *F. candida* fed on the non-*Bt* leaf-based diet containing E-64 ($\chi^2 = 32.17$, $P < 0.001$). Similarly, the mean body length and head width were not affected by ingestion of the *Bt* leaf-based diet ($P > 0.10$ for all sampling dates) (**Table 1**). In addition, the number of eggs produced per individual and the number of fecal pellets produced per individual was not affected by feeding on a *Bt* leaf-based diet (**Figure 2**) (Dunnett's tests; T1C-19 leaves: $P = 0.37$ for number of eggs, and $P = 0.98$ for a number of fecal pellets; T2A-1 leaves: $P = 0.093$ for number of eggs, and $P = 0.092$ for a number of fecal pellets). All of these parameters, however, were significantly reduced when *F. candida* fed on a non-*Bt* leaf-based diet containing E-64 (Dunnett's tests, all $P < 0.01$). As was the case with the pollen diets, the egg hatching rates of *F. candida* did not significantly differ among leaf-based diets (one-way ANOVA, $F_{3,147} = 1.73$, $P = 0.16$).

The r_m values of *F. candida* were 0.138 ± 0.004, 0.138 ± 0.001, 0.136 ± 0.003, and 0.117 ± 0.007 for *F. candida* that fed on a leaf-based diet from non-*Bt* plants, T1C-19 plants, T2A-1 plants, and non-*Bt* plants containing E-64, respectively. The mean r_m values did not significantly differ between *Bt* and non-*Bt* diet treatments (Dunnett's tests, $P = 1.0$ for T1C-19 leaves, $P = 0.969$ for T2A-1 leaves), except that the mean r_m value was significantly reduced when *F. candida* fed on the non-*Bt* diet containing E-64 ($P = 0.017$).

Concentrations of Cry Proteins by *F. candida* Feeding on *Bt* Rice Leaf Tissue

As indicated by ELISA measurements, *F. candida* that fed on a *Bt* rice leaf-based diet contained Cry proteins. The concentration of Cry1C detected in *F. candida* on days 7, 21, and 35 was 39.19 ± 0.24, 41.99 ± 1.09, and 64.60 ± 5.83 ng/g DW, respectively, when the collembolan fed on a T1C-19 leaf-based diet, and the concentration significantly increased over time (RM-ANOVA, $F = 458.1$, df = 3, $P < 0.001$). The concentration of Cry2A detected in *F. candida* on days 7, 21, and 35 was 147.53 ± 5.33, 140.08 ± 5.84, and 196.53 ± 0.19 ng/g DW, respectively, when the collembolan fed on a T2A-1 leaf-based diet, and the concentration significantly increased over time ($F_{1,3} = 7628957.0$, $P < 0.001$). No Cry protein was detected in *F. candida* that fed on a leaf-based diet from non-*Bt* rice plants.

Stability and Bioactivity of Cry Proteins in the Leaf-Based Diets

According to ELISA measurements, the original concentrations of Cry1C and Cry2A in the *Bt* rice leaf-based diets were

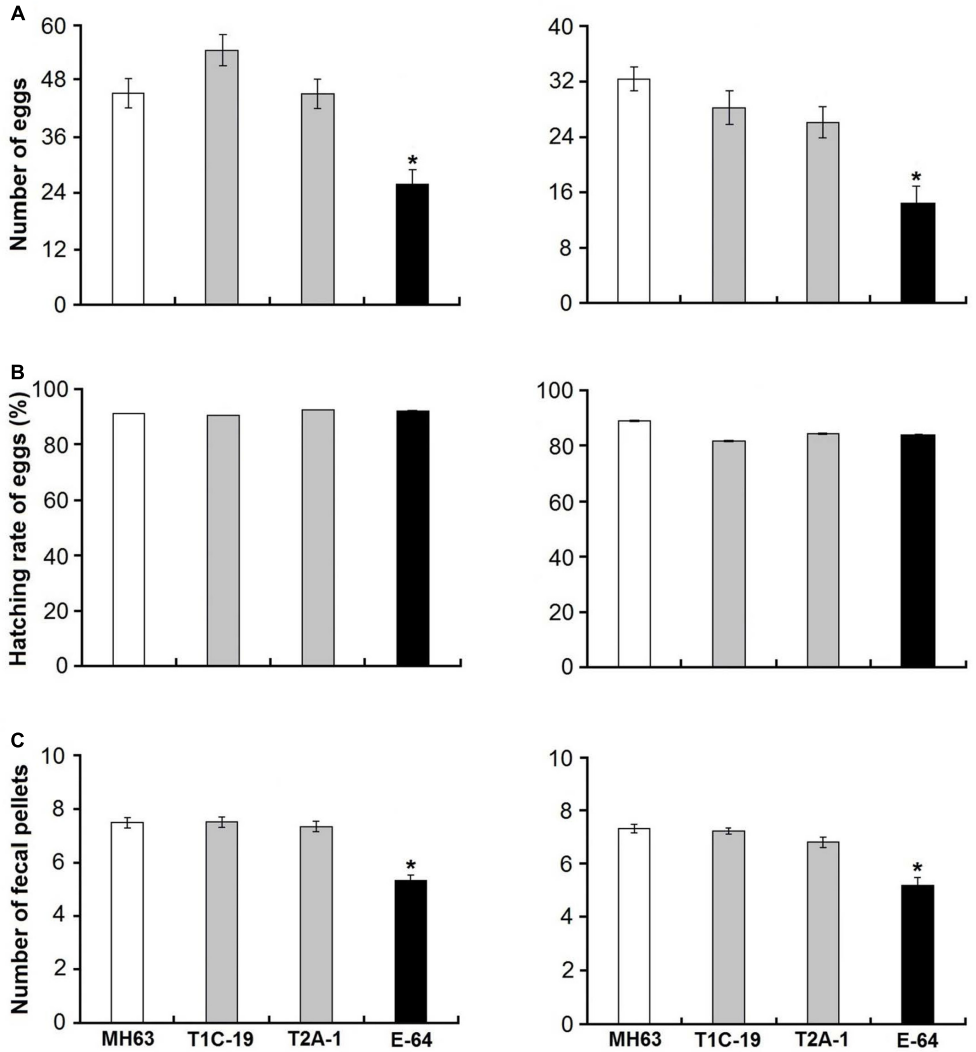

FIGURE 2 | Fecundity (numbers of eggs) produced per individual **(A)**, hatching rate of eggs **(B)**, and numbers of fecal pellets produced per individual **(C)** of *F. candida* fed on a diet consisting of rice pollen (left) or a mixture of rice leaf powder and baker's yeast (10:1) (right). The rice pollen and rice leaf powder were obtained from either of two *Bt* rice lines (T1C-19: Cry1C and T2A-1: Cry2A) or their non-*Bt* near isoline MH63 (negative control). For the positive control, the corresponding non-*Bt* rice tissue was supplemented with E-64 (positive control) for 35 days. Values are means ± SE, $n = 50$. Asterisks indicate a significant difference between the treatment and the negative control (MH63) (Dunnett's test, $P < 0.05$).

1.55 ± 0.03 and 16.38 ± 0.28 μg/g DW diet, respectively. After a 2-day feeding exposure, the contents had significantly decreased to 1.44 ± 0.01 and 13.64 ± 0.26 μg/g diet for Cry1C and Cry2A, respectively (Student's *t*-test; $t = 4.1$, df = 6, $P = 0.006$ for Cry1C, and $t = 7.1$, df = 6, $P < 0.001$ for Cry2A). No Cry protein was detected in the leaf-based diet made from non-*Bt* rice plant.

The sensitive-insect bioassay showed that the mortality of *C. suppressalis* larvae was 6.7 ± 3.3% when the larvae fed on a diet containing the extract from the non-*Bt* leaf-based diet for 7 days. The mortality was 90.0 ± 5.8% or 73.3 ± 3.3% when *C. suppressalis* larvae fed on diets containing the extract from a fresh Cry1C leaf-based diet (T1C-19) or from a Cry1C leaf-based diet that had been exposed to *F. candida* for 2 days. The mortality was 90.0 ± 5.8% or 80.0 ± 5.8% when *C. suppressalis* larvae were fed diets containing the extract from the fresh Cry2A leaf-based diet (T2A-1) or a Cry2A leaf-based diet that had been exposed to *F. candida* for 2 days. Mortalities were not significantly different for larvae that fed on fresh vs. 2-day-old diet (Chi-square test; $U = 2.78$, df = 1, $P = 0.10$ for Cry1C diet; $U = 1.18$, df = 1, $P = 0.28$ for Cry2A diet).

No Effects on Enzyme Activities in *F. candida* by Feeding *Bt* Rice Pollen or Leaf Tissue

The activity of the four enzymes did not significantly differ in *F. candida* that fed on diets containing *Bt* pollen vs. non-*Bt* pollen (**Figure 3**) or *Bt* leaf powder vs. non-*Bt* leaf powder (**Figure 4**)

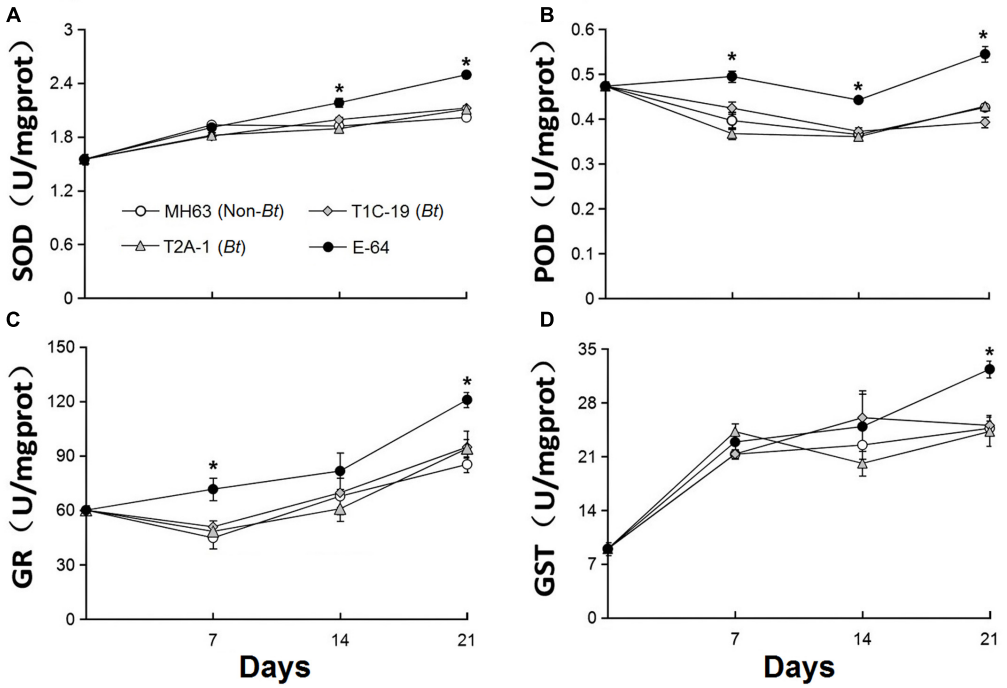

FIGURE 3 | Enzyme activities (**A**: peroxidase, POD; **B**: superoxide dismutase, SOD; **C**: glutathione, GR; and **D**: glutathione *S*-transferase, GST) in *F. candida* that fed on a diet consisting of non-*Bt* rice pollen (MH63, negative control), *Bt* rice pollen containing Cry1C or Cry2A (T1C-19 or T2A-1), or non-*Bt* rice pollen containing E-64 protein (positive control) for 35 days. Values are means ± SE, $n = 4$. Asterisks indicate a significant difference between the treatment and the negative control (MH63) ($P < 0.05$).

(RM-ANOVA, all $P > 0.05$). In contrast, the activities of the four enzymes were significantly higher ($P \leq 0.003$) in *F. candida* that fed on the non-*Bt* rice pollen containing E-64 rather than on non-*Bt* rice pollen without E-64 on all test days for POD, on days 14 and 21 for SOD, on days 7 and 21 for GR, and on day 21 for GST (**Figure 3**). The enzyme activities were also significantly higher ($P \leq 0.009$) in *F. candida* that fed on the non-*Bt* rice leaf-based diet containing E-64 rather than on the same diet without E-64 on all test days for SOD, on days 7 and 21 for POD, and on day 21 for GR and GST (**Figure 4**).

DISCUSSION

Pollen grains contain multiple organic and inorganic nutrients, such as sugars, starch, amino acids, proteins, lipids, vitamins, and minerals, and can serve as a food source for many arthropods (Li et al., 2010). Plant pollen has therefore been commonly used in dietary exposure assays with bees, lacewings, and ladybird beetles; such assays are essential components of non-target risk assessment of insect-resistant GE crops (Li et al., 2008, 2015; Wang et al., 2012, 2017; Meissle et al., 2014; Zhang et al., 2014). The current study shows that *F. candida* can survive, develop, and reproduce using rice pollen as a sole food source. The results are consistent with our previous study, in which *F. candida* survived well on only maize pollen (Zhang et al., 2017). In contrast, previous studies found that potato, cotton, and wheat leaves alone are not suitable foods for *F. candida* (Yu et al., 1997; Romeis et al., 2003; Bakonyi et al., 2006). Similarly, we found that the fitness of *F. candida* was significantly reduced when the collembolan fed on rice leaf powder alone (Yang et al., unpublished data). Based on our preliminary results, we developed a rice leaf-based diet in which baker's yeast was mixed with lyophilized leaf powder at a ratio of 1:10. The results indicate that when fed this diet formula, *F. candida* survival rates ≥88%, which meets the standard for such dietary exposure assays (Romeis et al., 2011).

Dietary exposure assays require an appropriate positive control to confirm that the assay is sensitive, i.e., to confirm that the assay can detect the toxic effects of a test compound (Li et al., 2014a). In the current study, E-64 was used as a positive control because it is readily accepted by *F. candida* and is known to be toxic to the collembolan (Zhang et al., 2017). Our feeding experiments showed that ingestion of *Bt* rice pollen or leaf powder from T1C-19 or T2A-1 rice plants did not reduce the survival, development, reproduction, or the intrinsic rate of natural increase (r_m) of *F. candida*. We found, however, that all of these life table parameters, except for the egg hatching rate, were significantly reduced by the consumption of the pollen or leaf-based diet containing E-64. This result demonstrates that the dietary exposure assays developed in our study were able to detect negative effects, and that they are therefore valid for assessing the effects of *Bt* rice pollen or leaf powder on *F. candida*. The results from the feeding bioassays thus indicate that consumption of *Bt* rice pollen or leaf powder has no adverse effects on *F. candida*

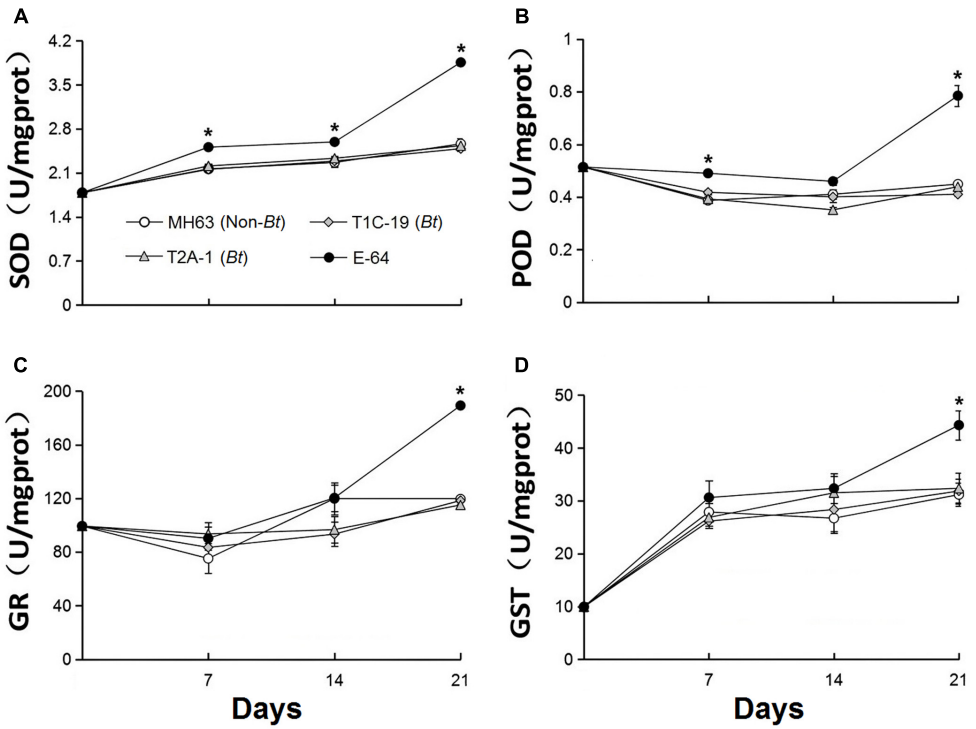

FIGURE 4 | Enzyme activities (**A**: POD, **B**: SOD, **C**: GR, and **D**: GST) in *F. candida* that fed on non-*Bt* rice leaves (MH63, negative control), *Bt* rice leaves containing Cry1C or Cry2A (T1C-19 or T2A-1), or non-*Bt* leaves containing E-64 protein (E-64, positive control) for 35 days. Rice leaf powder was mixed with baker's yeast (10:1). Values are means ± SE, $n = 4$. Asterisks indicate a significant difference between the treatment and the negative control (MH63) ($P < 0.05$).

individuals or populations. That E-64 did not reduce the egg hatching rate of *F. candida* that fed on the compound indicates that egg hatching is not a sensitive life-table parameter for assessing chemical toxicity to *F. candida*.

In addition to the life-table parameters mentioned above, the activities of two antioxidant-related enzymes, SOD and POD, and two detoxification-related enzymes, GR and GST, were measured, because they are known to be involved in the detoxification of reactive oxygen species (ROS) (Felton and Summers, 1995; Hayes and Strange, 2000). ROS might be induced when insects ingest toxic substances, and high levels of ROS may seriously damage the insects (Felton and Summers, 1995). It follows that an increase in the activity of these enzymes in insects may represent a response to the ingestion of a toxic, ROS-inducing compound. For these reasons, the activities of SOD, POD, GR, and GST have been widely used as indicators of the toxicity of *Bt* proteins and other insecticidal compounds (Bai et al., 2011; Yuan et al., 2011; Yang et al., 2015; Zhang et al., 2017). In the current study, the activities of SOD, POD, GR, and GST in *F. candida* were not affected by feeding on *Bt* rice pollen or leaf-based diets containing Cry1C or Cry2A protein. These results are consistent with previous studies. For example, the activities of SOD and POD in *F. candida* were not affected when the collembolan fed on the yeast mixed with Cry1Ab and Cry1Ac proteins (Yuan et al., 2011). Bai et al. (2011) found that SOD activity was not significantly altered in *F. candida* after ingestion of Cry1Ab-containing rice tissue for 35 days (Bai et al., 2011). Yang et al. (2015) showed that ingestion of pure Cry1C or Cry2A protein did not affect the activities of six enzymes in *F. candida* including antioxidant enzymes (SOD and POD), detoxification enzymes (GR and CES), and the proteases (T-Pro and TPS). More recently, Zhang et al. (2017) reported that SOD and POD were not influenced in *F. candida* that fed on the *Bt* corn pollen containing Cry1Ab/2Aj protein. In both Yang et al. (2015) and Zhang et al. (2017), the activities of these enzymes were significantly increased when *F. candida* ingested diets containing E-64, indicating that the assays used were able to detect toxic dietary effects. The lack of effects of consumption of *Bt* rice materials on life-table parameters further indicates that *F. candida* is not affected by Cry1C and Cry2A.

To quantify the exposure of *F. candida* to Cry protein in the feeding experiments, we measured the stability of the Cry proteins in the diets and the uptake of the proteins by *F. candida*. The results showed that the concentrations of Cry1C and Cry2A proteins in both pollen and leaf-based diets declined significantly during the feeding period, but that >60% of the Cry proteins was still detectable after a 2-day feeding exposure. The ingestion of Cry2A and Cry1C proteins by *F. candida* in the experiments was also confirmed by ELISA. In general, the contents of Cry proteins in *F. candida* increased over time with continually feeding on *Bt* pollen or leaf-based diets, which may be due to increased food consumption with *F. candida* growth. Furthermore, the bioactivity of the Cry proteins in the pollen or leaf-based diets was confirmed in a bioassay with *Bt* protein-sensitive *C. suppressalis*

larvae. These results demonstrate that *F. candida* larvae ingested bioactive Cry1C and Cry2A protein in our feeding experiments. Given that collembolans are soil organisms with a broad range of food (Ponge, 1991), the *F. candida* in our study, which were exclusively fed *Bt* rice material, were exposed to Cry proteins at levels much higher than would occur under field conditions. That no lethal or sublethal effects were detected under our worst-case exposure conditions demonstrates that *F. candida* is not sensitive to Cry1C or Cry2A proteins in *Bt* rice pollen and leaves. Our results also provide evidence that the genetic engineering of the rice plants has not resulted in any unintended or unexpected changes in rice that affect *F. candida* (Gong and Wang, 2013; Ladics et al., 2015; Schnell et al., 2015; Devos et al., 2016).

As a surrogate collembolan species, *F. candida* has been commonly used in non-target risk assessment of insecticidal GE plants including cotton, potato, wheat, maize, and rice (Yu et al., 1997; Romeis et al., 2003; Bitzer et al., 2005; Clark and Coats, 2006; Bai et al., 2011; Bakonyi et al., 2011; Yuan et al., 2011, 2013; Zhang et al., 2017). Most studies have reported that ingestion of *Bt* proteins or *Bt* protein-containing plant tissues did not have any adverse effects. Two exceptions are the studies by Bakonyi et al. (2006, 2011), in which *F. candida* produced significantly fewer fecal pellets after consuming powder from *Bt* (Cry1Ab) maize leaves rather than powder from non-*Bt* leaves. The reasons for this effect, however, were not elucidated. In summary, the available data with *F. candida* suggest that the currently used *Bt* Cry1, Cry2, and Cry3 proteins are not toxic to collembolans.

To our knowledge, the current report is the first to assess the potential effects of *Bt* rice pollen or leaves containing Cry1C or Cry2A proteins on *F. candida*. The results from our toxicological and biochemical experiments confirmed that Cry1C and Cry2A are not toxic to *F. candida*. The results also indicated the absence of unintended effects on the collembolan caused by any change in plant tissue nutritional composition due to foreign gene transformation. We therefore conclude that the planting of the *Bt* rice lines will pose a negligible risk to *F. candida*.

AUTHOR CONTRIBUTIONS

YL designed the study. BZ and YY performed all of the experiments. YY, YL, BZ, JR, and YP analyzed the data and wrote the manuscript. XZ and YP provided the experimental materials. All authors have read and approved the manuscript for publication.

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Overexpressing Exogenous 5-Enolpyruvylshikimate-3-Phosphate Synthase (EPSPS) Genes Increases Fecundity and Auxin Content of Transgenic Arabidopsis Plants

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Transgenic glyphosate-tolerant plants overproducing EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) may exhibit enhanced fitness in glyphosate-free environments. If so, introgression of transgenes overexpressing *EPSPS* into wild relative species may lead to increased competitiveness of crop-wild hybrids, resulting in unpredicted environmental impact. Assessing fitness effects of transgenes overexpressing *EPSPS* in a model plant species can help address this question, while elucidating how overproducing EPSPS affects the fitness-related traits of plants. We produced segregating T_2 and T_3 *Arabidopsis thaliana* lineages with or without a transgene overexpressing *EPSPS* isolated from rice or *Agrobacterium* (*CP4*). For each of the three transgenes, we compared glyphosate tolerance, some fitness-related traits, and auxin (indole-3-acetic acid) content in transgene-present, transgene-absent, empty vector (EV), and parental lineages in a common-garden experiment. We detected substantially increased glyphosate tolerance in T_2 plants of transgene-present lineages that overproduced EPSPS. We also documented significant increases in fecundity, which was associated with increased auxin content in T_3 transgene-present lineages containing rice *EPSPS* genes, compared with their segregating transgene-absent lineages, EV, and parental controls. Our results from Arabidopsis with nine transgenic events provide a strong support to the hypothesis that transgenic plants overproducing EPSPS can benefit from a fecundity advantage in glyphosate-free environments. Stimulated biosynthesis of auxin, an important plant growth hormone, by overproducing EPSPS may play a role in enhanced fecundity of the transgenic Arabidopsis plants. The obtained knowledge is useful for assessing environmental impact caused by introgression of transgenes overproducing EPSPS from any GE crop into populations of its wild relatives.

Keywords: abiotic stress, *Arabidopsis thaliana*, fitness, glyphosate-tolerance, growth hormone, indole-3-acetic acid, seed germination, transgenic plant

INTRODUCTION

Genetically engineered (GE) herbicide-tolerant crops are cultivated extensively over the world owing to their substantial agronomic, environmental, economic, health and social benefits (James, 2016). Herbicide-tolerant GE crops occupy ∼76% of the total GE crop cultivation area, including herbicide-tolerant and stacked herbicide-tolerant/insect-resistant GE crops (James, 2016). Of these, glyphosate-tolerance represents the world's most widespread GE crop trait (Duke and Powles, 2008; Vats, 2015; James, 2016). The commercial cultivation of glyphosate-tolerant GE crops has greatly promoted the glyphosate application in agricultural ecosystems, consequently arousing global concerns over its potential environmental impact. Many weed species have evolved glyphosate tolerance under selective pressure after long-term glyphosate applications (Duke and Powles, 2008; Délye et al., 2013). Glyphosate-selective-pressure induced target-site mutations (Gaines et al., 2010, 2011; Chen et al., 2015) and amplification of the *EPSPS* genes (Nandula et al., 2013; Sammons and Gaines, 2014) have been found in those resistant weeds resulting new environmental problems as farmers shift to less environmentally friendly herbicides. In addition, transgene flow from glyphosate-tolerant GE plants to populations of wild or weedy relatives has been found, becoming an environmental biosafety concern (Reichman et al., 2006; Warwick et al., 2008; Wegier et al., 2011; Zapiola and Mallory-Smith, 2017). Yet, some researchers posit little or no environmental impact from introgression of glyphosate-tolerance transgenes into wild relative populations because they believe that such transgenes offer no fitness advantage in natural ecosystems in the absence of glyphosate (Cerdeira and Duke, 2006; Vila-Aiub et al., 2014).

The findings of significantly enhanced fecundity of crop-weed (Wang et al., 2014) and crop-wild (Yang et al., 2017b) rice hybrid progeny containing the glyphosate-tolerance transgene in a glyphosate-absent habitat suggests that introgression of such a glyphosate-tolerance transgene might result in transgene persistence and spread in populations of wild relatives, possibly causing environmental consequences (Qiu, 2013; Ryffel, 2014; Vila-Aiub et al., 2015; Martin et al., 2017). Glyphosate can competitively inhibit EPSPS (5-enolpyruvylshikimate-3-phosphate synthase, EC 2.5.1.19), resulting in weakness or even death of plants at the proper dosages (Roberts et al., 1998; Mueller et al., 2003; Duke and Powles, 2008). EPSPS is a key enzyme in the shikimate pathway, which is extremely important because ∼35% or more plant biomass in the form of dry matter is represented by aromatic molecules derived directly from this pathway (Franz et al., 1997). In addition, EPSPS is essential for the production of aromatic amino acids (e.g., tryptophan, phenylalanine, and tyrosine) and other secondary metabolites (Weaver and Herrmann, 1997), suggesting that EPSPS is crucial for the survival, growth, and development of plants.

Overproduction of EPSPS, attempting to provide sufficient surplus of EPSPS binding to glyphosate to reduce its fatal toxicity, is one of the strategies to increase plants' tolerance to glyphosate. This strategy includes developing GE plants with multiple copies of the *EPSPS* gene (Rogers et al., 1983; Goldsbrough et al., 1990; Shyr et al., 1992) and increasing basal levels of the EPSPS enzyme by fusing an *EPSPS* gene with a strong promotor (Klee et al., 1987; Su et al., 2008). Notably, certain GE glyphosate-tolerant crops overexpressing *EPSPS* not only have increased glyphosate tolerance (Klee et al., 1987; Su et al., 2008; Vats, 2015; Yang et al., 2017a), but unexpectedly also showed increased yield (Zhou et al., 2003; Owen et al., 2010) and other fitness traits. The increased yield can also be manifest as increased fecundity in GE hybrid progeny with weedy (*O. sativa* f. *spontanea*, Wang et al., 2014) and wild (*O. rufipogon*, Yang et al., 2017b) rice overexpressing *EPSPS*, even under glyphosate-free conditions. Wang et al. (2014) also reported significantly increased Trp concentrations in crop-weed F_2 transgene-present hybrid lineages of the GE rice (*Oryza sativa*) line overexpressing *EPSPS* and four weed rice populations in the glyphosate-free environment. It has been proven that Trp is associated with the biosynthesis of plant growth hormone auxin (Zhao, 2012). In addition, Yang et al. (2017b) reported considerably altered phenology of F_2 *EPSPS* transgene-present hybrid lineages with two wild rice populations in the glyphosate-free environment. Altogether, these findings suggest that transgenes overproducing EPSPS will change fitness of crop-weed/wild hybrids.

Gressel et al. (2014) and Grunewald and Bury (2014) questioned whether the enhanced fecundity and metabolic traits in the glyphosate-tolerant crop-weed hybrids overexpressing *EPSPS* was due to the position effect of the gene insertion or the possible linkage with neighboring sequences because only a single transgenic event was involved in the study of Wang et al. (2014). It is therefore critical to confirm the observed increases in fecundity and metabolic traits are caused by the *EPSPS* transgene *per-se*, rather than the other actions of transgenes. In addition, it is necessary to address whether the observed changes in fecundity by overexpression of the *EPSPS* transgene is a general phenomenon? In other words, can the phenomenon observed in rice (*Oryza*) be also found in very distant plant species such as Arabidopsis? To answer the above question, we produced multiple independent events of transgenic *Arabidopsis thaliana* plants overexpressing exogenous *EPSPS* genes from different sources (rice and *Agrobacterium*), driven by a cauliflower mosaic virus 35S promotor (pCaMV35S). We also produced transgenic plants only containing the selective marker gene (*nptII*) driven by pCaMV35S as the empty vector (EV) control. The T_2 and T_3 segregating transgene-present and transgene-absent lineages, EV and parent controls were compared to estimate differences in glyphosate tolerance and fitness-related traits (**Figure 1**) to test whether the observed changes in rice would also occur in a phylogenetically distant plant species.

The objectives of this study are to address the following questions: (1) Does overproduction of EPSPS increase glyphosate tolerance of the Arabidopsis plants in transgenic lineages? (2) Does overexpression of exogenous *EPSPS* genes enhance fitness of transgenic Arabidopsis plants? (3) If so, is enhanced fitness of the transgenic plants caused by the position effect of an inserted gene? (4) Do transgene-present lineages that overproduce EPSPS synthesize more auxin than transgene-absent lineages? Answers to these questions will increase our understanding of the general effects of overexpressing *EPSPS* genes on the phenotypes of plant species.

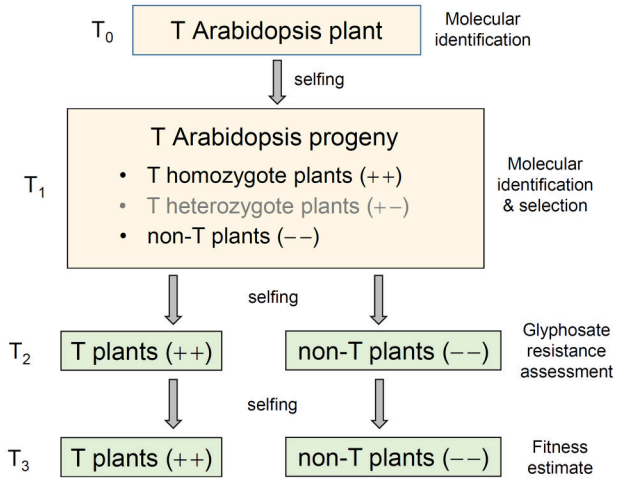

FIGURE 1 | A schematic pedigree to illustrate the production of transgenic (T) Arabidopsis progeny containing genes overexpressing the 5-enolpyruvoylshikimate-3-phosphate synthase (EPSPS). A T_0 transgenic Arabidopsis plant was self-pollinated to produce the T_1 progeny that contained transgene-homozygote (+ +), transgene-heterozygote (+ −), and transgene-absent (− −) plants. The isogenic transgene-homozygote (+ +) and transgene-absent (− −) lineages generated from T_1 plants through self-pollination (selfing) and molecular identification were retained for the experiments of glyphosate tolerance (T_2), and *EPSPS* transgene expression, biomass and auxin (IAA) content, and fitness assessment (T_3).

MATERIALS AND METHODS

Arabidopsis thaliana Transgenic Lineages

An *A. thaliana* strain Columbia (Col 0, coded as P) was used as the parent to produce the comparative transgene-present and transgene-absent lineages in this study. Three *EPSPS* genes were used to develop transgenic constructs: two isolated from cultivated rice (Zhou et al., 2006; Su et al., 2008) and one (*CP4*, coded as C) from an *Agrobacterium* sp. strain (Padgette et al., 1996). The two genes from rice included one normal *EPSPS* (coded as E, Zhou et al., 2006) and another mutant *EPSPS* that was a C^{317}-T mutation (coded as Em, Su et al., 2008). This Em gene was obtained by an error-prone PCR (polymerase chain reaction) technique and an Em transgenic rice line conferred high level of tolerance to glyphosate (Su et al., 2008; Lu et al., 2014a,b). The vector pCHF3 was used for overexpression transgenic constructs that contained one of the target *EPSPS* genes and a selective marker gene (*nptII*), both driven by pCaMV35S, respectively (Figure S1, upper panel). The *nptII* (neomycin phosphotransferase) marker gene conferred tolerance to kanamycin. An empty vector only including the selective marker gene (*nptII*) driven by pCaMV35S was also developed (Figure S1, lower panel) as a control.

The transgenic constructs were introduced into *Agrobacterium tumefaciens* first and then transformed into Arabidopsis plants by the floral-dip method (Clough and Bent, 1998) for genetic transformation. All seeds from the transformation-treated Arabidopsis plants were germinated on the 1/2 MS culture media, including 30 g sucrose, 2.2 g M519 (Murashige & Skoog Basal Medium with Vitamins), 50 ng kanamycin, and 8 g agar per liter (pH 5.7). All survived plants (T_0) from the 1/2 MS culture media were subjected to molecular identification for the target and marker transgenes ($Appendix S2) to confirm their transgenic status. The primer sequences for the *EPSPS* and mutant *EPSPS* transgenes were 5′-acgaatgagggagagaccga-3′ and 5′-accatcagcgaagagtgcaa-3′; whereas those for the *CP4* transgene were 5′-gcgtcgccgatgaaggtgctgt-3′ and 5′-cggtccttcatgttcggcggtctc-3′. Primer sequences for the *nptII* marker gene were 5′-taaagcacgaggaagcggtc-3′ and 5′-gatggattgcacgcaggttc-3′. All the primers were synthesized by the Shanghai Sangon Biotech Co., Ltd (Shanghai, China). To retain transgenic plants that are presumed to have a single copy of the transgenes (E, Em, or C), we selected T_1 progeny generated from self-pollination of a T_0 transgenic plant with a 3: 1 segregation ratio for kanamycin tolerant: sensitive, and confirmed by molecular identification (Figure S2) for further experiments.

To determine the transgene insertion or position effect causing the fitness change, we retained three transgenic events from each of the three transgenic constructs (E, Em, or C) with the above-average level of *EPSPS* expression for each transgenic construct in the T_2 and T_3 generations for further analyses (Appendix S3). The reason for measuring transgenic events in T_2 and T_3 was to examine the stability of *EPSPS* expression between generations. Transgenic events with the highest level of *EPSPS* expression in the T_2 and T_3 generations (Table S1) were excluded from the experiment to avoid the extreme cases. We only retained the isogenic lineages with transgenic homozygote (+ +) and transgene-absent plants (− −) from T_1 segregating populations after molecular identification and selection for the transgenes in the T_2 generation (**Figure 1**, Appendix S2). A total of 20 Arabidopsis lineages, including three transgene-present lineages (E+, Em+, and C+) for each of the three events (9), three segregated transgene-absent lineages (E−, Em−, and C−) for each of the three events each representing the three transgenes (9), one empty vector lineage (EV), and one parental strain (P), were used for further experiments.

Glyphosate Tolerance

Twenty Arabidopsis lineages in the T_2 generation (**Figure 1**) were used to test glyphosate (Roundup®, glyphosate isopropyl amine salt aqueous solution: 41%) tolerance in a dose-response experiment. For each lineage (event, EV, or parent), three replicates ($n = 3$) each included three plants grown in a growth chamber (22°C), were included. A total of 180 plants were included in the test for glyphosate tolerance for the 20 lineages at each glyphosate concentration. Forty days after seeds were sown, all plants were sprayed with glyphosate aquatic solution at nine different concentrations: 0, 0.1, 0.2, 0.4, 0.8, 1.0, 1.2, 2.0, 5.0 m*M*, in which 0.4 m*M* equivalent to the concentration of 840 g/ha is the commonly used dosage for glyphosate application in fields for the agricultural weed control (Norsworthy et al., 2008). Therefore, a total of 1,620 plants was used in the glyphosate tolerance experiment. Survival ratios were determined as the number of surviving Arabidopsis plants as percent of the total

number of plants used for analyses 15 days after glyphosate application. Plants that were partially green but with green meristems and central rosettes were scored as "surviving;" plants that had white meristems and central rosettes were scored as "dead" (Figure S3).

EPSPS Transgene Expression and EPSPS Protein Content

Twenty Arabidopsis lineages in the T_3 generation (**Figure 1**) were used to determine *EPSPS* transgene expression and EPSPS protein content. Each Arabidopsis plant in the T_3 generation was equally divided as two parts for measuring *EPSPS* transgene expression and the EPSPS protein content, respectively. For each of the 20 lineages (6 E+ and E–, 6 Em+ and Em–, 6 C+ and C–, 1 EV and 1 P), a pooled sample (each including 8 plants) with three replicates ($n = 3$) were used for these measurements 30 days after seeds were sown. Therefore, a total of 480 plants was used in this experiment.

Real-time PCR was applied to determine the expression of *EPSPS* transgene relative to an *ubiquitin* reference gene (*UBQ*) from Arabidopsis. Total RNA was isolated from the 30-day-old plants using the RNAsimple Total RNA kit (TianGen, Beijing, China). DNA removal and RNA reverse-transcription were conducted using the PrimeScript® RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). The primers for real-time PCR were designed using the software Primer PREMIER ver. 5.0. The primer sequences for the *EPSPS* and mutant *EPSPS* transgenes were 5′-aaggatgcgaaagagg-3′ and 5′-caacccgacaaccaa-3′; whereas those for the *CP4* transgene were 5′-tggattgcgatgaggg-3′ and 5′-tgatcgagatgggtggc-3′. The primer sequences for the reference gene (*UBQ*) were 5′-aatgtgaaggcgaagatccaagac-3′ and 5′-agacggaggacgagatgaagc-3′. The expression of the *EPSPS* genes in non-transgenic, EV, and parental lineages was measured using the Arabidopsis endogenous *EPSPS* gene. The primer sequences for the Arabidopsis endogenous *EPSPS* gene were 5′-aacgcaagttatgtcc-3′ and 5′-gcagttagtgccaag-3′. All the primers were synthesized by the Shanghai Sangon Biotech Co., Ltd. (Shanghai, China). The real-time PCR reaction mixture kit (TaKaRa, Dalian, China) included 1 μl of template cDNA, 0.4 μl each of the forward and reverse 10 μM primers, and 1 × SYBR®Premix Ex Taq™ in a final volume of 20 μl. PCR reaction was conducted at 94°C 30 s; 94°C 15 s, 60°C 15 s, and 40 s at 72°C for 40 cycles.

The sandwich technique of ELISA (enzyme linked immunosorbent assay) was performed to determine the EPSPS protein content. The total proteins were extracted based on the method of phosphate-buffered saline (PBS, 28.7 g Na_2HPO_4-$12H_2O$ and 2.96 g NaH_2PO_4-$2H_2O$, pH 7.4) with 10% (v/v) methanol in 0.1 M PBS to suspend the samples after ground into powder in liquid nitrogen. The samples were then incubated in ice bath for 40 min, followed by centrifuged at 8,000 × g, at 4°C for half an hour. The supernatant was collected for EPSPS protein content determination with the Quantiplate kit (Envirologix, Portland, OR, USA) for detecting EPSPS protein in plants and bacteria following the manufacturer's instructions. The Microplate Reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to detect the optical density (OD) at the wavelength of 450 nm.

Fitness-Related Traits

Twenty Arabidopsis lineages in the T_3 generation were used to estimate fitness (**Figure 1**). Nine fitness-related traits were used for the measurement at different times: seed germination under normal and stressed conditions, leaf area, plant height and branching, the number of siliques per plant, number of seeds per silique and per plant (Table S2). To estimate seed germination ratios for each lineage, six replicate samples ($n = 6$) each with 50 seeds were germinated on 1/2 MS culture media under the normal, heat, and drought stress conditions, respectively (Table S2). To estimate the other fitness-related traits (Appendix S5), six replicates ($n = 6$) each with four plants were grown in each of the 20 lineages in a growth chamber (22°C). The layout of the total 120 replicates (pots) followed a completely randomized design.

Biomass and Auxin Content

Six Arabidopsis lineages with or without the *EPSPS* transgenes (E, Em, and C) in the T_3 generation (**Figure 1**; Table S1) were used to measure biomass and auxin content. The auxin content was determined as the average total weight (ng) of auxin in plants at the same growth stage. One transgenic event (E2, Em3, or C2) each representing one of the three transgenic constructs (see Table S1) was randomly selected to measure plant biomasses and the auxin content, using fresh samples of the entire 30-day-old plants. From each event, five independent replicates ($n = 5$), each represented by five different plants of transgene-present or transgene-absent lineages, were measured for the biomass and auxin content (a pooled sample from five plants per replicate, Table S2), following the methods of Chen et al. (2012). A total of 150 plants were used for determining biomasses and the auxin content. The empty vector and parental lineages were not included for analyses.

Statistical Analyses

One way ANOVA was conducted to examine the effect of the presence or absence of each *EPSPS* transgene, involving transgene-present (E+, Em+, or C+), transgene-absent (E–, Em–, or C–), EV, and parental lineages. One way ANOVA was also conducted to determine the effect of different events from each transgenic construct (E, Em, or C) on fitness-related traits. Duncan's multiple range test was conducted to determine significant differences in gene expression, protein content, and the fitness-related traits that showed the significant transgenic effect among transgene-present, transgene-absent, EV, and parental lineages based on one way ANOVA. The independent *t*-tests with Bonferroni corrections were conducted to test for significant differences in all measured fitness-related traits and auxin content between transgene-present and transgene-absent lineages, after the measured values were subject to the Levene's test for equality of variances. All statistical analyses were performed using the software SPSS ver. 19.0 (IBM Inc., New York, USA).

TABLE 1 | Average survival ratios (%) of *Arabidopsis thaliana* plants in T_2 transgene-present (E+, Em+, or C+) lineages overexpressing *EPSPS* and their segregating transgene-absent (E–, Em–, or C–), empty vector (EV), and parental (P) lineages under different glyphosate concentrations.

Event/ lineage	Survival ratio (%) of Arabidopsis plants under different glyphosate concentration (m*M*)								
	0	**0.1**	**0.2**	**0.4**[a]	**0.8**	**1.0**	**1.2**	**2.0**	**5.0**
E+/2	100	100	100	**100**	100	88.9 (11.1)	77.8 (11.1)	0	0
E–/2	100	100	55.6 (11.1)	**0**	0	0	0	0	0
E+/3	100	100	100	**100**	88.9 (11.1)	66.7 (19.2)	55.6 (11.1)	0	0
E–/3	100	100	66.7 (19.2)	**0**	0	0	0	0	0
E+/4	100	100	100	**100**	88.9 (11.1)	77.8 (11.1)	55.6 (11.1)	0	0
E–/4	100	100	77.8 (11.1)	**0**	0	0	0	0	0
Em+/2	100	100	100	**100**	100	88.9 (11.1)	77.8 (11.11)	0	0
Em–/2	100	100	66.7 (0)	**0**	0	0	0	0	0
Em+/3	100	100	100	**100**	100	77.8 (11.1)	66.7 (19.25)	0	0
Em–/3	100	100	66.7 (19.2)	**0**	0	0	0	0	0
Em+/4	100	100	100	**100**	88.9 (11.1)	77.8 (11.1)	55.6 (11.1)	0	0
Em–/4	100	100	33.3 (19.2)	**0**	0	0	0	0	0
C+/2	100	100	100	**100**	100	100	88.9 (11.1)	22.2 (11.1)	0
C–/2	100	100	55.6 (11.1)	**0**	0	0	0	0	0
C+/3	100	100	100	**100**	88.9 (11.1)	66.7 (19.2)	66.7 (19.2)	0	0
C–/3	100	100	77.8 (11.1)	**0**	0	0	0	0	0
C+/4	100	100	100	**100**	100	88.9 (11.1)	66.7 (19.2)	0	0
C–/4	100	100	55.6 (11.1)	**0**	0	0	0	0	0
EV	100	100	59.3 (9.1)	**0**	0	0	0	0	0
P	100	100	62.9 (10.6)	**0**	0	0	0	0	0

Numbers in parenthesis indicate standard errors (n = 3 replicates with three plants per replicate).

[a] *0.4 mM (bold fonts) is equivalent to the concentration of 840 g ae/ha, which is the commonly used dosage for glyphosate application in fields for agricultural weed control (Norsworthy et al., 2008).*

RESULTS

Glyphosate Tolerance

Substantially increased tolerance to glyphosate was detected in Arabidopsis plants of transgene-present lineages of all events representing the three transgenic constructs, compared to those of their transgene-absent, EV, and parental lineages in the T_2 generation (**Table 1**; Figure S3a,b). The three types of transgenic Arabidopsis plants (E+, Em+, and C+) survived at different concentrations (0.1–1.2 m*M*) of glyphosate, although with some degrees of variation (∼10–35%) among transgenic constructs at the same concentration. More than 50% transgenic plants of the three constructs survived when the concentration of glyphosate increased to 1.2 m*M* (**Table 1**). However, none of the transgene-absent Arabidopsis plants (E–, Em–, and C–) survived at the concentration of 0.4 m*M* glyphosate (**Table 1**), which was equivalent to the commonly applied glyphosate dosage (840 g/ha) in the field. Notably, ∼7% plants in the transgenic *CP4* lineages (C+) survived at the concentration of 2.0 m*M* glyphosate.

Expression of *epsps* Transgenes and EPSPS Protein Content

Significantly increased expression of the three *EPSPS* transgenic constructs (E, Em, and C) and content of the EPSPS proteins were detected in Arabidopsis plants of transgene-present lineages, compared to those of transgene-absent, EV, and the parental lineages (**Table 2**). The average values of relative expression of the three transgenes: *EPSPS*, mutant *EPSPS*, and *CP4*, measured by real-time PCR were significantly greater ($P < 0.01$) in the T_3 transgene-present lineages (E+, Em+, and C+) than those in their corresponding transgene-absent (E–, Em–, and C–), the EV, and parental lineages based on the Duncan's multiple range test (**Table 2**). In addition, the average values of EPSPS protein content measured by ELISA were also significantly greater ($P < 0.05$) in the T_3 transgene-present lineages (E+, Em+, and C+) than those in their corresponding transgene-absent (E–, Em–, and C–), the EV and parental lineages based on the Duncan's multiple range test (**Table 2**).

Fecundity and Other Fitness-Related Traits

We first tested differences in all included fitness-related traits among the three transgenic events based on each transgene-present or transgene-absent lineage using one-way ANOVA. Because none of these traits showed significant differences among the transgenic events ($P < 0.05$, Table S3), we grouped data from three events each with six replicates ($n = 18$) for each transgenic construct in subsequent analyses (Table S4). Thus, for seed germination, we analyzed data from 1,800 seeds per transgene for each treatment, while for other fitness-related traits, we analyzed data from 144 plants per transgene. One-way ANOVAs indicated significant effects of the presence versus absence of the three transgenes (E, Em, or C) on some fitness-related traits in the

TABLE 2 | Means of relative transgene expression and EPSPS protein content in T_3 *Arabidopsis thaliana* plants of transgene-present (E+, Em+, and C+), transgene-absent (E–, Em–, and C–), empty vector (EV), and the parental lineages, measured by real-time PCR (polymerase chain reaction) and ELISA (enzyme linked immunosorbent assay), respectively.

Lineage	Relative gene expression in different events[1]			Protein content (ng/ml) in different events[2]		
Event ID:	**2**	**3**	**4**	**2**	**3**	**4**
EPSPS						
E+	**6.609 (0.943)**^{A}	4.354 (0.320)^{A}	3.022 (0.353)^{A}	199.48 (0.66)^{a}	198.76 (0.14)^{a}	202.64 (0.80)^{a}
E–	**0.012 (0.002)**^{B}	0.007 (0.001)^{B}	0.006 (0.001)^{B}	146.74 (1.33)^{c}	149.68 (0.52)^{c}	156.06 (1.04)^{c}
EV	0.010 (0.004)^{B}	0.010 (0.004)^{B}	0.010 (0.004)^{B}	178.26 (0.43)^{b}	178.26 (0.43)^{b}	178.26 (0.43)^{b}
P	0.008 (0.002)^{B}	0.008 (0.002)^{B}	0.008 (0.002)^{B}	177.51 (3.10)^{b}	177.51 (3.10)^{b}	177.51 (3.10)^{b}
MUTANT *EPSPS*						
Em+	6.008 (0.824)^{A}	**4.725 (0.734)**^{A}	1.000 (0.479)^{A}	219.91 (0.38)^{a}	228.26 (0.14)^{a}	219.91 (0.80)^{a}
Em–	0.054 (0.015)^{B}	**0.035 (0.002)**^{B}	0.018 (0.001)^{B}	182.21 (0.25)^{b}	183.65 (1.88)^{b}	176.74 (0.88)^{b}
EV	0.010 (0.004)^{B}	0.010 (0.004)^{B}	0.010 (0.004)^{B}	178.26 (0.43)^{b}	178.26 (0.43)^{b}	178.26 (0.43)^{b}
P	0.008 (0.002)^{B}	0.008 (0.002)^{B}	0.008 (0.002)^{B}	177.51 (3.10)^{b}	177.51 (3.10)^{b}	177.51 (3.10)^{b}
CP4						
C+	**6.585 (0.476)**^{A}	4.228 (0.226)^{A}	1.331 (0.321)^{A}	211.23 (0.99)^{a}	205.66 (1.01)^{a}	209.41 (0.25)^{a}
C–	**0.026 (0.005)**^{B}	0.018 (0.001)^{B}	0.014 (0.003)^{B}	178.26 (0.50)^{b}	182.14 (0.90)^{b}	176.54 (0.66)^{b}
EV	0.010 (0.004)^{B}	0.010 (0.004)^{B}	0.010 (0.004)^{B}	178.26 (0.43)^{b}	178.26 (0.43)^{b}	178.26 (0.43)^{b}
P	0.008 (0.002)^{B}	0.008 (0.002)^{B}	0.008 (0.002)^{B}	177.51 (3.10)^{b}	177.51 (3.10)^{b}	177.51 (3.10)^{b}

Numbers in parenthesis indicate standard errors (n = 3 replicates, each including eight different plants as a pooled sample). Events with bold letters indicate those used for biomass and auxin examination.

[1]Different capital letters indicate significances at the 0.01 level. [2]Different small letters indicate significances at the 0.05 level.

TABLE 3 | One-way ANOVA to test the effects of each of the three *epsps* transgenes on fitness-related traits in T_3 *Arabidopsis thaliana* plants, including the transgene-present (3), transgene-absent (3), empty vector (1), and parental (1) lineages.

Trait	***EPSPS* (E)**			***Mutant EPSPS* (Em)**			***CP4* (C)**		
	df	***F***	***P***	**df**	***F***	***P***	**df**	***F***	***P***
Seed germination under normal condition	3	1.84	0.218	3	0.43	0.737	3	1.61	0.262
Seed germination under heat stress	3	21.83	**<0.001**	3	12.86	**<0.001**	3	0.67	0.576
Seed germination under drought stress	3	15.66	**0.001**	3	14.08	**0.001**	3	0.87	0.495
Relative leaf area	3	8.40	**<0.001**	3	5.32	**0.003**	3	3.85	**0.016**
Plant height at maturity	3	3.92	**0.014**	3	1.33	0.278	3	3.14	**0.035**
Number of branches per plant	3	0.98	0.412	3	1.32	0.279	3	0.96	0.420
Number of siliques per plant	3	5.95	**0.002**	3	6.36	**0.001**	3	5.22	**0.004**
Number of seeds per silique	3	2.01	0.126	3	2.96	**0.042**	3	1.08	0.366
Number of seeds per plant	3	7.23	**<0.001**	3	9.46	**<0.001**	3	5.19	**0.004**

For seed germination, each lineage included six replicates (n = 6) each containing 50 seeds. For other traits, each lineage included six replicates (n = 6) each containing four plants. Bold values indicate the significant traits.

T_3 generation (**Table 3**), including relative leaf area, plant height, and the numbers of siliques and seeds per plant (**Table 3**).

All of these significant differences were in the direction of increased fitness for the transgene-present lineages relative to the controls. For example, overproduction of EPSPS was associated with increases of ∼12–22% ($n = 18$) more seeds to germinate under the heat and drought stresses for the E and Em transgene-present lineages (**Figures 2A–F**; Table S4), although no significant differences in seed germination under the normal condition (22°C) (Table S4). Notably, the C+ transgene-present lineage showed a significant increase in seed germination under the drought stress (**Figure 2F**). In addition, overproduction of EPSPS resulted in 22–28% ($n = 18$) more siliques and 23–27% ($n = 18$) more seeds per plant (**Figures 3A–F**; Table S4). The number of branches per plant and seeds per silique was very similar across lineages (Table S4). Overall, the presence of the three transgenes was associated with larger plants (**Figures 4A–F**; Table S4) with enhanced fecundity (**Figures 3A–F**; Table S4) in the glyphosate-free environment. We did not detect evidence of fitness benefits or costs associated with the single EV lineage, compared to the parental and transgene-absent lineages.

Biomass and Auxin Content

Significant increases in biomass and auxin content were detected in 30-day-old plants in transgene-present E+ and Em+ lineages in the T_3 generation (**Figures 5A, B**). However,

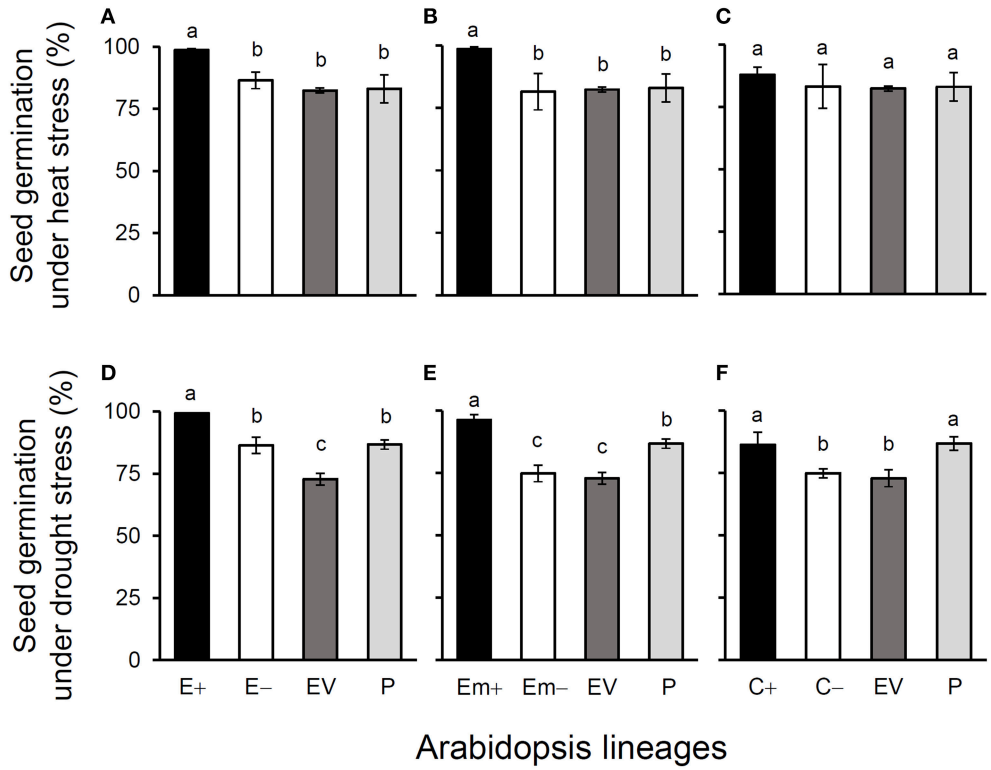

FIGURE 2 | Average % seed germination of three Arabidopsis transgenic events under heat **(A–C)** and drought **(D–F)** stresses in T_3 transgene-present, transgene-absent, empty vector (EV), and parent (P) lineages in the glyphosate-free environment. Different letters above the columns indicate significances at $P < 0.05$ based on Duncan's multiple range test ($n = 18$). E+: *EPSPS* transgene-present lineages, E–: *EPSPS* transgene-absent lineages, Em+: mutant *EPSPS* transgene-present lineages, Em–: mutant *EPSPS* transgene-absent lineages; C+: *CP4* transgene-present lineages, C–: *CP4* transgene-absent lineages. Bars represent standard errors.

no significant differences in biomass or auxin content were observed between transgene-present C+ and transgene-absent C– lineages, although the values were slight higher for the C+ lineage. About 28–33% increases in biomass were observed in the transgene-present E+ and Em+ lineages, compared with their transgene-absent counterparts (**Figure 5A**). Increases in auxin content of around 25–33% were detected in transgene-present E+ and Em+ lineages, compared with their segregating transgene-absent counterparts (**Figure 5B**).

DISCUSSION

Results from this study demonstrated that the transfer of overexpressing *EPSPS* genes isolated from different sources, namely rice (E or Em) and *Agrobacterium* (*CP4*), into *A. thaliana* plants substantially increased their tolerance to the glyphosate (Roundup®) herbicide although with considerable variation among the three *EPSPS* transgenes. This finding is based on the dose-response comparisons of >1,600 Arabidopsis plants among transgene-present lineages, their isogenic transgene-absent counterparts, the empty vector (EV), and parent controls in a climate chamber. In the dose-response experiment, all transgene-present Arabidopsis plants survived at the 0.4 m*M* glyphosate dosage, which is equivalent to the commonly used concentration of 840 g/ha for glyphosate application for agricultural weed control (Norsworthy et al., 2008). In contrast, none of the Arabidopsis plants in the corresponding transgene-absent lineages, EV, and parent controls survived at this dosage (0.4 m*M*). Increased glyphosate tolerance has also been observed in other GE plants overexpressing *EPSPS* transgenes, such as *Petunia hybrida* (Shah et al., 1986), rice (Su et al., 2008; Lu et al., 2014a,b), tobacco plants (Jones et al., 1996), and *A. thaliana* (Klee et al., 1987; Yang et al., 2017a), regardless of the origins (endogenous or exogenous) of *EPSPS* genes. All these results indicate that the transfer of an overexpressing *EPSPS* gene into plants, including the model plant Arabidopsis in this study and other studies (Klee et al., 1987; Yang et al., 2017a), can increase glyphosate tolerance of the transgenic plants due to overproduction of EPSPS.

Increased tolerance to glyphosate in our transgenic plants overexpressing an *EPSPS* gene is presumably due to the sufficiently surplus EPSPS that can bind glyphosate (Rogers et al., 1983), as reported in *P. hybrida* (Shah et al., 1986), tobacco plants (Jones et al., 1996), and Arabidopsis (Klee et al., 1987; Yang et al., 2017a). Thus, these results confirm the strategy of overproducing EPSPS driven by a strong promoter (e.g., pCaMV35S for dicots and pUbiquitin for monocots) to be effective in increase GE crops' tolerance to glyphosate, regardless of exogenous (as in

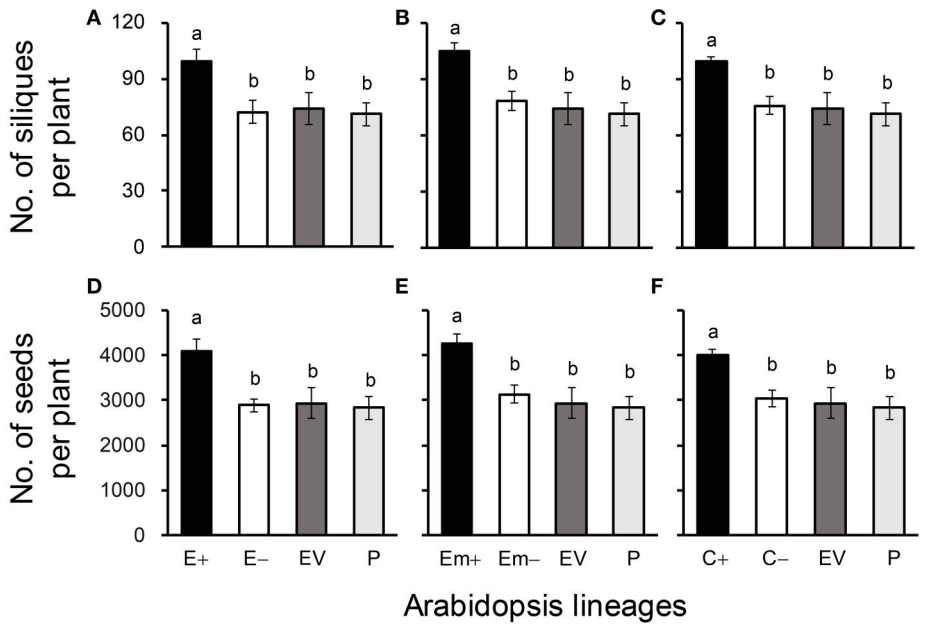

FIGURE 3 | Average silique **(A–C)** and seed production **(D–F)** of three Arabidopsis transgenic events in T_3 transgene-present, transgene-absent, empty vector (EV), and parent (P) lineages in the glyphosate-free environment. Different letters above the columns indicate significances at $P < 0.05$ based on Duncan's multiple range test ($n = 18$). E+: *EPSPS* transgene-present lineages, E–: *EPSPS* transgene-absent lineages, Em+: mutant *EPSPS* transgene-present lineages, Em–: mutant *EPSPS* transgene-absent lineages; C+: *CP4* transgene-present lineages, C–: *CP4* transgene-absent lineages. Bars represent standard errors.

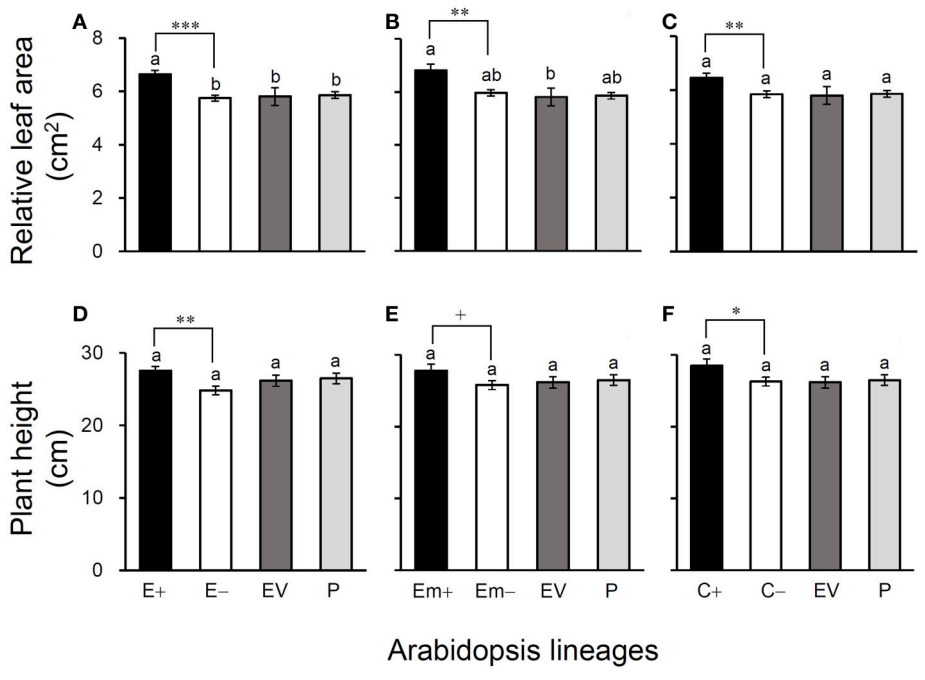

FIGURE 4 | Average relative leaf-area **(A–C)** and plant height **(D–F)** of three Arabidopsis transgenic events in T_3 transgene-present, transgene-absent, empty vector (EV), and parent (P) lineages in the glyphosate-free environment. Different letters above the columns indicate significances at $P < 0.05$ based on Duncan's multiple range test ($n = 18$). Differences between transgene-present and transgene-absent lineages were compared based on the independent *t*-test after Bonferroni correction ($n = 18$). $+P < 0.1$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. E+: *EPSPS* transgene-present lineages, E–: *EPSPS* transgene-absent lineages, Em+: mutant *EPSPS* transgene-present lineages, Em–: mutant *EPSPS* transgene-absent lineages; C+: *CP4* transgene-present lineages, C–: *CP4* transgene-absent lineages. Bars represent standard errors.

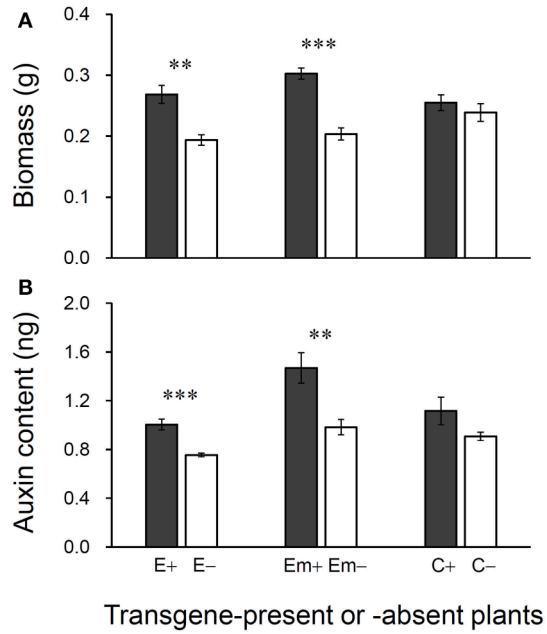

FIGURE 5 | Differences in biomass **(A)** and auxin (IAA) content **(B)** of 30-day plants between T_3 transgene-present and transgene-absent Arabidopsis lineages based on the independent *t*-test ($n = 5$). E+: *EPSPS* transgene-present lineage, E–: *EPSPS* transgene-absent lineages (event E/2); Em+: mutant *EPSPS* transgene-present lineages, Em–: mutant *EPSPS* transgene-absent lineages (event Em/3); C+: *CP4* transgene-present lineages, C–: *CP4* transgene-absent lineages (event C/2). $^{**}P < 0.01$, $^{***}P < 0.001$. Bars represent standard errors.

this study) or endogenous (Klee et al., 1987; Su et al., 2008; Yang et al., 2017a). The application of an endogenous transgene overproducing EPSPS at a proper level to develop GE glyphosate-tolerant crops may have particular commercial values. That is, a glyphosate-tolerance transgene originating from the crop species rather than a bacterium or other sources may reduce consumers' concerns over the food safety issues (Kuiper et al., 2001; Qaim and Zilberman, 2003). In addition, the application would be particularly useful for GE crops such as sugar beets, potatoes, and vegetables, of which the consumed parts are vegetative organs, because the transgenic plants appear to have increased vigor, but with less opportunities of crop-to-wild/weed gene flow mediated by pollination.

As often happens in studies of transgenic plants, we also observed considerable variation in overexpression of the *EPSPS* genes among different transgenic events in our experiment, based on the real-time PCR analysis. Therefore, we only included transgenic events with a relatively high level of *EPSPS* expression because the main objective of this study was to determine whether overexpression of *EPSPS* genes would enhance fecundity of transgene-present plants. To address the question about the likelihood of insertion or position effect by a single transgenic event with enhanced fecundity of transgenic plants (Gressel et al., 2014; Grunewald and Bury, 2014), we included GE Arabidopsis plants of three transgenic events representing each of the three transgenic constructs with the above-average level of *EPSPS* overexpressing in our common-garden experiment for fitness comparisons. Our results did not show significant differences in fitness-related traits among the three included transgenic events of each transgene in transgene-present Arabidopsis lineages. In addition, we did not find significant differences in fitness-related traits between the EV and parental lineages. These results support previous findings that enhanced fitness/fecundity (as shown in (Su et al., 2008; Wang et al., 2014; Yang et al., 2017a),b) is not the consequence of an insertion or position effect (process of transgenesis), but due to the action of the transgene itself. We therefore confirm that overexpression of *EPSPS* in GE plants with a proper level can result in overproduction of their EPSPS and increased glyphosate tolerance.

In the common-garden experiment in a growth chamber, we observed significantly increased seed germination ratios in E+ and Em+ transgene-present lineages when seeds were exposed to the heat (28°C) and drought [200 m*M* D-mannitol ($C_6H_{14}O_6$)] stresses, although no differences were found in seed germination among different lines when seeds were exposed to the normal temperature (22°C, ideal for Arabidopsis, Xiong et al., 1999). The *CP4*+ transgene-present lineages only showed significant increases in seed germination under the drought stress. Considering that auxin (IAA) can promote seed germination and plant growth under abiotic stresses (Woodward and Bartel, 2005; Liu et al., 2014; Naser and Shani, 2016), our explanation for enhanced seed germination under stresses can be attributed to the increased auxin content in transgenic Arabidopsis plants. The report of Leadem (1987) in which auxin stimulated seed germination under special conditions including heat and cold stresses supports our explanation. However, we propose more studies to test this hypothesis because very limited examples are found in the scientific literature.

In addition, our results indicated significantly greater values of a few major fitness-related traits, including the number of siliques and seeds per plant in transgene-present Arabidopsis lineages in glyphosate-free environment. Similar findings of increased fitness were also reported in transgene-present Arabidopsis plants containing a native gene overproducing EPSPS (Beres et al., in press). All these findings support our previous observation of the significantly enhanced fecundity in transgene-present crop-weed (Wang et al., 2014) and crop-wild (Yang et al., 2017b) hybrid lineages containing an *EPSPS* transgene from rice. It is apparent that the presence of a transgene overproducing EPSPS, regardless of its origin (endogenous or exogenous), may significantly enhance the fecundity of a plant. Altogether, findings from wild/weedy rice (monocot) and Arabidopsis (eudicot) indicate that overexpression of an *EPSPS* gene to a proper level with increased fecundity of GE plants in the glyphosate-free environments may be a general feature of angiosperms. Therefore, environmental impact caused by introgression of a transgene overexpressing *EPSPS* from GE glyphosate-tolerant crops into their wild/weedy relatives should be thoroughly assessed, even in the glyphosate-free environment. Further studies including hybrid descendants of transgenic crops overexpressing *EPSPS* with their wild relatives should be

conducted to provide more evidence for the potential ecological impact.

What could be the underlying mechanisms for enhanced fecundity of GE plants containing an *EPSPS* overexpressing transgene in glyphosate-free environment? In this study, we detected increased auxin, an important plant growth hormone (Woodward and Bartel, 2005; Zhao, 2012; Liu et al., 2014), in transgene-present Arabidopsis lineages (E+ and Em+). We therefore hypothesize that increased total endogenous auxin may play a role in promoting the growth and development (probably also stress tolerance) of transgene-present Arabidopsis plants, eventually leading to increases in their fitness-related traits, although other factors such as enhanced photosynthetic rates by overproducing EPSPS (see Wang et al., 2014) can also promote the growth of transgene-present plants. As indicated in our previous study, significantly increased tryptophan (Trp) content was detected in four independent transgene-present crop-weed hybrid lineages overproducing EPSPS (Wang et al., 2014). Trp is an aromatic amino acid synthesized in the downstream of EPSPS in the shikimate pathway (Herrmann and Weaver, 1999; Maeda and Dudareva, 2012). Recent studies have revealed that auxin biosynthesis is a simple two-step pathway converting Trp to auxin in plants (Mashiguchi et al., 2011; Won et al., 2011). This suggests that overproduction of EPSPS may lead to increases in auxin through increased Trp (Zhao, 2012; Wang et al., 2014). Thus, the complete discovery of the precise biosynthesis pathway from EPSPS to auxin will provide deeper insight into mechanisms associated with fitness effect and environmental impact of transgenic plants that overproduce EPSPS in agricultural and natural habitats.

AUTHOR CONTRIBUTIONS

JF: Conducted the experiment, analyzed data, and wrote the paper; PN, ZG, XG, and Y-QF: Conducted some part of the experiment; B-RL: Conceived and designed the experiment, analyzed data, and wrote the paper. All authors reviewed 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/fpls.2018.00233/full#supplementary-material

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Lack of Detection of Bt Sugarcane Cry1Ab and NptII DNA and Proteins in Sugarcane Processing Products Including Raw Sugar

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Brazil is the largest sugarcane producer and the main sugar exporter in the world. The industrial processes applied by Brazilian mills are very efficient in producing highly purified sugar and ethanol. Literature presents evidence of lack of DNA/protein in these products, regardless of the nature of sugarcane used as raw material. Recently CTNBio, the Brazilian biosafety authority, has approved the first biotechnology-derived sugarcane variety for cultivation, event CTC175-A, which expresses the Cry1Ab protein to control the sugarcane borer (*Diatraea saccharalis*). The event also expresses neomycin-phosphotransferase type II (NptII) protein used as selectable marker during the transformation process. Because of the high purity of sugar and ethanol produced from genetically modified sugarcane, these end-products should potentially be classified as "pure substances, chemically defined," by Brazilian Biosafety Law No. 11.105. If this classification is to be adopted, these substances are not considered as "GMO derivatives" and fall out of the scope of Law No. 11.105. In order to assess sugar composition and quality, we evaluate Cry1Ab and NptII expression in several sugarcane tissues and in several fractions from laboratory-scale processing of event CTC175-A for the presence of these heterologous proteins as well as for the presence of traces of recombinant DNA. The results of these studies show that CTC175-A presents high expression of Cry1Ab in leaves and barely detectable expression of heterologous proteins in stalks. We also evaluated the presence of ribulose-1,5-bisphosphate carboxylase/oxygenase protein and DNA in the fractions of the industrial processing of conventional Brazilian sugarcane cultivars. Results from both laboratory and industrial processing were concordant, demonstrating that DNA and protein are not detected in the clarified juice and downstream processed fractions, including ethanol and raw sugar, indicating that protein and DNA are removed and/or degraded during processing. In conclusion, the processing of conventional sugarcane and CTC175-A Bt event results in downstream products with no detectable concentrations of heterologous DNA or new protein. These results help in the classification of sugar and ethanol derived from CTC175-A event as pure, chemically defined substances in Brazil and may relieve regulatory burdens in countries that import Brazilian sugar.

Keywords: sugar, highly purified substance, sugarcane, Cry1Ab, neomycin-phosphotransferase type II

INTRODUCTION

Brazil is the largest sugarcane producer and sugar exporter in the world. With an estimated planted area of 9.1 million ha and a total annual yield of 694.54 million tons of sugarcane, Brazil produces an estimated 39.8 million tons of sugar almost entirely devoted to use as a food ingredient. Ethanol fuel production for the domestic and international markets is also an important use of Brazilian sugarcane, representing half of total annual sugarcane yield. Agricultural biotechnology has been used widely in Brazil for almost 20 years in crops such as soybeans, maize, and cotton and recently the Brazilian biosafety authority CTNBio has approved the first biotechnology-derived sugarcane variety for cultivation.

Sugarcane yield is negatively impacted by pests and diseases typically seen in tropical cultivation conditions. A major insect pest impacting Brazilian sugarcane production is the sugarcane borer (*Diatraea saccharalis*). Infestation by this pest has been shown to reduce shoots, tillers, and plant weight, increase lodging, produce drying of young spindle leaves, and allow infections by opportunistic microorganisms, including bacteria and fungi. Yield losses in excess of 10% and a negative impact on sugar quality (increased levels of secondary metabolites such as dextrans and poor color characteristics) are common as a result of borer infestation (Precetti and Téran, 1983; Precetti et al., 1988; Botelho and Macedo, 2002). Centro de Tecnologia Canavieira (CTC), one of the major suppliers of adapted sugarcane germplasms in Brazil, has developed event CTB141175/01-A (abbreviated here as CTC175-A), which expresses the Cry1Ab protein in leaf tissue to control the sugarcane borer. The event also expresses the neomycin-phosphotransferase type II (NptII) protein used as selectable marker during transformation process. The food/feed and environmental safety of event CTC175-A was extensively evaluated by CTNBio, the Brazilian regulatory authority. Vegetative "seed cane" propagation has begun in controlled field conditions leading to commercial sugar production in Brazil in the 2020 timeframe.

Sugar is extracted from sugarcane stalks which are pressed to produce the sugarcane juice. OECD states that the extracted juice has high water content (about 85%) and contains mainly sucrose and reducing sugars (RSs) like glucose and fructose and that its protein content is negligible, around 0.2% of the dry matter (OECD, 2011). Additionally, sugarcane processing involves harsh conditions known to precipitate and denature protein and DNA, leading to the removal of detectable intact plant DNA and protein in raw and refined sugar (Cullis et al., 2014).

Industrial production of sugar from sugarcane involves extraction of sugarcane juice, clarification, concentration, crystallization, centrifugation, and sugar drying. The sugar processing can be classified as white sugar production and raw sugar production. White sugar can be produced directly from sugarcane if harsh a clarification step is employed. Alternatively, and more usually, white sugar is produced from an additional refining step of raw sugar (Brokensha, 1998). In raw sugar production, juice is physically extracted from the sugarcane by pressing stalks using either a tandem roller mill or diffuser mill. Cut cane pieces are first shredded, immersed in water and then crushed between sets of rollers to release the primary juice (tandem mill); alternatively, shredded cane is extensively rinsed and percolated with recycling ~80°C water to obtain the primary juice (diffuser mill). The residual fibrous material (Bagasse) is typically dried and used as boiler fuel and the surplus is burned to produce electric energy sold to the public grid. In the second phase, primary juice is filtered and clarified by heating at 105°C for 3 h in the presence of lime (calcium hydroxide) and/or a flocculent to precipitate plant macromolecules (protein, DNA, fiber, etc.). The resulting heavy precipitate, called "mud," forms which is separated from the juice in the clarifier, and then filtered to produce filter cake which is removed. The resulting clarified juice (14–20°Brix) is concentrated by vacuum evaporation, at an initial temperature of approximately 110°C which then is decreased to 85–90°C, with concomitant increase of vacuum. This evaporation step finishes when syrup of around 65°Brix is produced (Hugot, 1969; Bruijn, 1998). This syrup is concentrated, at 70°C, in a vacuum evaporative crystallizer to produce raw sugar. The first round of sugar crystallization is performed in around 2–3 h but the process can be repeated several times until no more sucrose crystallizes (Hugot, 1969; Bruijn, 1998). The residual liquid called molasses is mixed with sugarcane juice and yeast and fermented to produce ethanol. After recovery of the ethanol the residual fermentation solids are removed by centrifugation to yield vinasse which is typically used as fertilizer. The next process is the refining of the raw sugar to refined sugar which is the final food ingredient (**Figure 1**).

Therefore, the processes of extraction, raw sugar production, and refining involves multiple steps involving conditions known to denature, precipitate, and eliminate DNA and protein macromolecules found in low concentrations in sugarcane stalks (Cheavegatti-Gianotto et al., 2011; Cullis et al., 2014). As a result, OECD states that sugar is a very purified substance as raw sugar is typically 97–98% sucrose, whereas refined sugar purity is about 99.93% sucrose. The remaining impurities in refined sugar are water, inverted or reducing sugars (glucose and fructose), ash, colored components, and other organic non-sugar compounds (OECD, 2011).

Unlikely other sugarcane producer countries, in Brazil, molasses is almost entirely used for biofuel production, and Brazilian mills do not produce alcoholic beverages, known as "rum," from this residue. A Brazilian sugarcane spirit, known as *cachaça* or *aguardente*, is produced directly from fresh fermented sugarcane juice, in industrial or artisanal facilities which are distinct from sugarcane mills devoted to sugar and ethanol production. In those facilities, after being extracted, the juice is fermented by yeasts to produce the "wine." This wine is then boiled in copper stills giving rise to vapors that are then condensed by cooling producing a liquid with high alcohol content (38–54°GL). The liquid obtained in the initial distillation phase is discarded due to the presence of compounds that are more volatile than ethanol. The last fraction of distillation is also discarded due the presence of low volatile substances. In practice, only the middle fraction of distillation, representing 75–85%, is used for consumption. After distillation, this fraction is filtered and consumed directly or after aged in wood barrels. The vast majority of this cachaça production is devoted to domestic market. The steps of boiling and distilling required for cachaça production are likely to remove traces of protein and DNA from the final product.

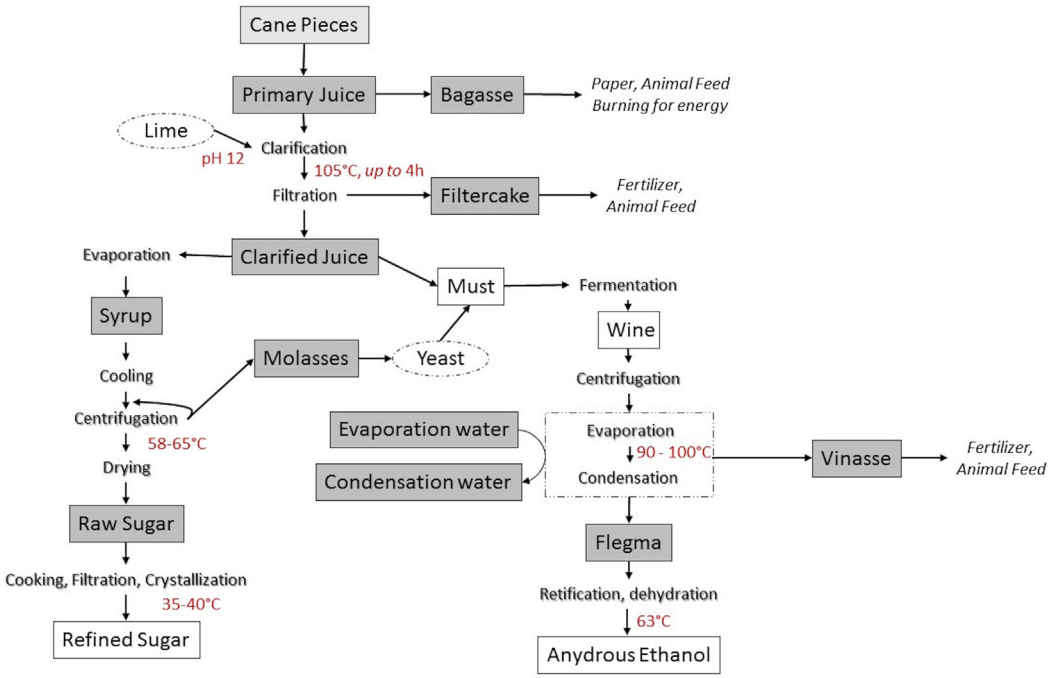

FIGURE 1 | Sugarcane industrial processing and derived food ingredients. Gray boxes indicate sampling points in the process. Lighter gray box (cane pieces) indicates the sampling of leaves.

Due to extremely harsh conditions of sugarcane processing and the resulting purity of those substances, sugar and ethanol produced from all sugarcane, including genetically modified sugarcane, should potentially be classified as "pure substances, chemically defined," by Brazilian Biosafety Law No. 11.105. One of the requirements for this classification is that the substance should not have the GMO itself, neither heterologous protein/DNA in its final composition. If this classification is to be adopted, these substances are not considered as "GMO derivatives" according to Brazilian Biosafety Law. Additionally, this information is important for importer countries to evaluate the food safety of sugar derived from CTC175-A event. The rationale behind the food risk assessment it that the absence, or presence at extremely low levels of heterologous protein in the article of commerce (sugar) would lead to extremely high consumption safety margins due to none/very low exposure to the heterologous protein. By the scientific point of view, this information, in conjunction with the well established safety of Cry1Ab and NptII proteins, should lessen the safety concerns of using sugar derived from CTC175-A as a food ingredient (Kennedy et al., *in this issue*).

Several experiments, described here, were conducted on conventional sugarcane or on event CTC175-A in Brazil. Specifically, studies evaluated the original expression levels of Cry1Ab and NptII in CTC175-A tissues, and the fate of total protein, Cry1Ab and NptII protein and DNA during processing of event CTC175-A sugarcane. Other studies on conventional sugarcane examined the effects of processing on ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), DNA, and protein. Results indicate that CTC175-A expresses heterologous proteins in very low levels at the sugarcane juice, the raw material for sugar and ethanol production, and that sugarcane processing degrades/removes protein and DNA leading to the production of sugar and ethanol in which these substances are not identified by conventional detection techniques.

MATERIALS AND METHODS

Sugarcane Event CTC175-A Expression Cassettes and Newly Expressed Proteins

Event CTC175-A sugarcane was obtained using biolistic plant transformation, by inserting a DNA fragment containing the expression cassettes for the *cry1Ab* and *nptII* genes into sugarcane variety CTC20, a commercially grown conventional variety cultivated in the Center-South region of Brazil. The DNA fragment used in transformation contains the expression cassettes of the *cry1Ab* gene, which encodes a 648-amino acid *Bacillus thuringiensis* protein, and the *nptII* gene, which encodes 263 amino acid type II neomycin phosphotransferase (**Figure 2**).

Cry1Ab is a well-studied insecticidal protein, which confers resistance to certain lepidopteran pests including the sugarcane borer (*D. saccharalis*), while NptII is used as a selectable marker used in the transformation process that confers resistance to aminoglycoside-type antibiotics such as neomycin. The expression of the *cry1Ab* and *nptII* genes is regulated by the promoters of the corn Pepcarboxylase gene (PEPC) and the ubiquitin gene of the corn (ubi-1), respectively. Both genes utilize the nopaline synthase terminator (NOS), from *Agrobacterium tumefaciens*.

FIGURE 2 | Transformation cassette used to obtain CTC175-A event *via* biolistic transformation of CTC20 sugarcane variety.

The *cry1Ab* gene present in event CTC175-A corresponds to a synthetic and truncated DNA sequence (Koziel et al., 1992). This sequence had its nucleotides synthetically optimized using preferred codons to enhance expression in corn. The *nptII* gene is derived from the Tn5 transposon of *Escherichia coli* (Fraley et al., 1983).

Sugarcane Field Agronomic Management

In order to comply with Normative Resolution No. 05 from CTNBio (*Comissão Técnica Nacional de Biossegurança*—Brazilian Technical Biosafety Commission) which requires evaluation of environmental, food, and feed biosafety, and to analyze its phenotypic performance, the event CTC175-A was planted in six locations representative of the crop area of the progenitor cultivar CTC20 in Brazilian Center-South (Paranavaí—Paraná State, Uberlândia—Minas Gerais State, Montividiu—Goiás State; Conchal, Piracicaba, and Jaboticabal—São Paulo State), in the season 2014/2015.

In each location, standard agronomic practices for sugarcane cultivation (soil preparation, fertilization, pest management) were applied evenly throughout the experiment. Treatments (event CTC175-A and the conventional CTC20) were allocated within each block, forming the plots or experimental units. Each plot was represented by four rows of 10 m spaced by 1.5 m adding up an area of 6.0 m × 10.0 m. The experiments were arranged in a randomized complete block-design with 4 replications. In order to assess Cry1Ab and NptII levels at a time representative of harvest and processing to sugar, tissue samples were collected 365 days after planting in field experiments. All experimental fields were conducted under the official CTNBio approvals obtained through compliance with Normative Resolution No. 06.

Evaluation of Cry1Ab and NptII Expressions in CTC175-A Tissues

In order to evaluate expression levels of proteins Cry1Ab and NptII in CTC175-A event, samples of leaves, stalks, and roots were collected in all replicates from all site experiments and immediately frozen on dry ice until laboratory evaluation. Samples were processed by grinding on dry ice to a fine powder. Protein extractions were performed on representative aliquots of the processed samples. ELISA methodology was used to quantify the proteins in sample extracts.

Cry1Ab protein was extracted from the sugarcane plant tissue samples using the tissue extraction protocol and quantitative assay protocol that follows. An aliquot of each tissue sample was weighed (approximately 15–20 mg) into a 2.0 mL tube. Stainless steel beads were added to each tube. Using buffer ratios of 10:1, an appropriate volume of ELISA extraction buffer (1.5 mL of phosphate-buffered saline with Tween20) was added to each sample. Tissues were pulverized in a Geno Grinder 2010 for approximately 2.5 min at a frequency of 290 *g*. Samples were incubated at 4–8°C for approximately 15 min. Extracts were spun down at ≥12,350 *g* for 10 min at 4°C in a microcentrifuge. Approximately 1.0 mL of the supernatant was collected and placed in a fresh 2.0 mL centrifuge tube. Supernatant from the sample extraction was diluted in deionized water to fall within the range of the standard curve. Remaining supernatants were then frozen at −20°C. The presence of the Cry1Ab protein was detected using a validated ELISA (EnviroLogix Qualiplate Cry1Ab ELISA kit).

Neomycin-phosphotransferase type II protein was extracted from the sugarcane plant tissue samples using the tissue extraction protocol and quantitative assay protocol that follows. An aliquot of each tissue sample was weighed (approximately 45–55 mg for leaf tissue and 190–210 mg for root and internode tissue) into a 2.0 mL tube. Four stainless steel beads were added to each tube. An appropriate volume of ELISA extraction buffer was added to each sample. 1.5 mL of 1× PEB (supplied with kit) was used. Tissues were pulverized in a Geno Grinder 2010 for approximately 2.5 min at a frequency of 290 *g*. Samples were incubated at 4–8°C for approximately 15 min. Extracts were spun down at 12,350 *g* for 10 min at 4°C in a microcentrifuge. Approximately 1.0 mL of the supernatant was collected and placed in a fresh 2.0 mL centrifuge tube. Supernatant from the sample extraction was diluted in 1× PEB to fall within the range of the standard curve. Remaining supernatants were then frozen at −20°C. The presence of the NptII protein was detected using a validated ELISA assay (Agdia NptII ELISA Kit).

Control sample extracts were analyzed concurrently to confirm the absence of plant-matrix effects in ELISA. For each ELISA, a standard curve was generated with known amounts of the corresponding reference protein. Cry1Ab protein standard calibrators at 100, 75, 50, 25, 12.5, 6.25, 3.13, and 0 ng/mL were prepared in deionized water. NptII protein standard calibrators at 20, 15, 10, 5, 2.5, 1.25, 0.625, and 0 ng/mL were prepared in PBST. Calibrators were prepared fresh each day from a working stock solution. The mean absorbance for each sample extract was plotted against the appropriate standard curve to obtain the amount of protein as nanograms per milliliter (ng/mL) of extract. The concentrations were converted to represent the amount of protein as micrograms per gram (μg/g) of tissue by the following formula:

$$(\text{ng/mL}) \times (\text{dilution factor}) \times (\text{volume of buffer [mL]}) / (\text{amount of tissue[g]}) \times 1{,}000.$$

The predetermined extraction efficiencies were used to adjust the transgenic protein concentrations to the estimated total

concentration in the corresponding tissue sample by the following formula:

$$\text{amount of protein measured from a single extraction } (\mu g/g) / \text{extraction efficiency } (\%).$$

All calculations, including mean and SD, were performed with Microsoft Excel® 2007 spreadsheet software. All decimal places associated with the concentrations determined for each replicate sample were used in calculation of the mean, where were then rounded to two decimal places for reporting consistency.

CTC175-A and CTC20-Derived Sugar and Ethanol Production at Laboratory Scale to Evaluate DNA and Protein Loss

In order to comply with Brazilian Biosafety Normatives for regulated genetically modified plant material, one batch of sugar from CTC175-A and one batch of sugar from CTC20 were produced. Mature stalks of CTC175-A event sugarcane and the parental conventional variety CTC20 were collected from all plots of the experiment planted at Piracicaba/SP at 365 days after planting and processed into raw sugar and ethanol using laboratory scale methods (Novello, 2015; Merheb, 2014; Merheb et al., 2016) that mimic the industrial processes used by Brazilian mills. Harvested stalks were immediately transported to the laboratory for sugarcane juice extraction and subsequent processing to collect process fractions including raw sugar.

Approximately 56.0 L of sugarcane juice was extracted from 90 stalks of each variety by shredding and pressing in the laboratory. Leaf, fiber, and sugarcane juice samples were collected from each variety for DNA, protein, and sugar quality analyses. The stalk quality of sugarcane varieties was evaluated by analyses of fiber, starch, brix, dextran, RSs, total RSs, pH, polarization, and purity (**Table 1**). These characteristics are factors that have a direct impact on the quality of the final products and the yields of the processes (Santos et al., 2012).

The sugarcane juices of CTC175-A and CTC20 were heated to 70°C with constant stirring, immediately after reaching 70°C, the juice was neutralized (pH 7) by adding lime. Following neutralization, juice was further heated to 98–100°C for approximately 2 min, and then transferred to a vessel, containing approximately 3 ppm of anionic polymer flocculant (Flonex 9076)/liter of juice. Following flocculation and decantation, clarified juice (supernatant) was separated from the sludge and samples were collected.

Clarified juice was concentrated from 20 to 65° Brix to generate syrup, using a rotary evaporator. After concentration, this syrup was used in crystallization which was performed using a laboratory reactor (Marconi MA 502), with an 8.0 L internal volume, that was equipped with a helical-type agitator. After the preparation of syrup, 1.0 L of syrup was added in crystallizer to be concentrated from 65 to 84° Brix in vacuum (22in Hg). At this point, 30 g of refined sugar were seeded. Afterward, in the same vacuum, the crystallizer feeding was performed by a controller. When feeding stopped, the crystallizer was in standby for 90 min, and the final evaporation started to be concentrated from 84 to 90° Brix in vacuum. After 6 h, vacuum was removed and the mass was centrifuged and washed with steam. The resultant dense mass of sugar crystals was centrifuged using a laboratory basket centrifuge (Metalúrgica Sueg Ltda), with a capacity of 1.0 kg of crystal sugar per batch (Merheb, 2014; Merheb et al., 2016). In these experiments, approximately 1.0 kg of sugar was produced per crystallization. Following centrifugation, sugar crystals were air-dried for approximately 12 h (Merheb et al., 2016).

TABLE 1 | Methodologies used for analyzing characteristics used for sugar classification in Brazilian market.

Characteristic	Methodology	Reference
Starch	Starch—determination in raw sugar	ICUMSA Method GS 1-16 (2009)
Ash	The determination of conductivity ash in raw sugar, brown sugar, juice, syrup, and molasses	ICUMSA – Método GS 1/3/4/7/8-13 (1994)
Color	Determination of solution color of raw sugars brown sugars and colored syrups at pH 7.0	ICUMSA – Method GS 9/1/2/3-8 (2011c)
Dextran	The determination of dextran in raw sugar by a modified alcohol haze method	ICUMSA Method GS 1/2/9-15 (2011b)
Filterability	Método BR-SM-PR-103	Supplemental Methodology S1
Acid Floc	Método BR-SM-PR-420	Supplemental Methodology S1
Alcohol Floc	Método BR-SM-PR-271	Supplemental Methodology S1
RS	Method 32—reducing sugars—determination in raw sugar by the Lane and Eynon method	The Laboratory Manual for Australian Sugar Mills (2001)
Polarization	The determination of the polarization of raw sugar by polarimetry	ICUMSA GS 1/2/3/9-1 (2011a)
Turbidity	Methods of analysis—formazin turbidity standards	ASBC (1976)
Sugars	High-performance liquid chromatography (HPLC)	Sluiter et al. (2006)

Vinasse and Flegma (diluted ethanol) were obtained from the juice in a single cycle batch fermentation performed in triplicate using must composed of sugarcane juice with approximately 160 g/L TRS and 100 g/L fresh PE-2 industrial yeast in a final fermentation volume of 500 mL. Fermentation was performed in an Erlenmeyer flask placed in a shaker (Innova 44, New Brunswick Scientific) at 0.805 *g* and 32°C for 8h. Simple distillation was performed to separate flegma resulting from fermentation from the vinasse, using a distiller (Tecnal Redutec TE–086 alcohol microdistiller). The wine was heated to 90–100°C for 5 min for flegma evaporation and condensation. Vinasse was the distillation residue. For ethanol production, it is necessary to use a distillation column to purify the flegma into ethanol.

All sugar production and sugar analysis were performed in laboratories certified with CQB (*Certificado de Qualidade em* Biossegurança—Biosafety Quality Certification) granted by CTNBio according to Normative Resolution No. 01. All personnel working in these activities were trained according the requirements of Normative Resolution No. 02 for contained activities with genetically modified plant material.

Compositional Analysis of Sugar Produced in Laboratory

Raw sugars and other common parameters were analyzed at CTC's laboratories certified with CQB to comply with Brazilian biosafety requirements. The quality of the raw sugar produced from either event CTC175-A or CTC20 conventional was assessed for sugar quality parameters: starch, ash, color, dextran, filterability, acid floc, alcohol floc, RSs, polarization, turbidity, and sugars (sucrose, glucose, fructose). The analytical methodologies used to classify sugar according to Brazilian market are presented in **Table 1** and are relevant for sugar classification and placement to specific markets in Brazil (Oliveira et al., 2007a,b).

DNA Detection in Laboratory Sugar Fractions

To evaluate the fate of DNA and proteins at the different laboratory processing stages, samples were collected during the processing of sugarcane to raw sugar and ethanol. Both solid samples (leaf, bagasse, and sugar) and liquid samples (primary juice, clarified juice, sludge, syrup, molasses, flegma, and vinasse), were collected from both cultivars (CTC175-A event and CTC20 isoline). The DNA extraction protocol used was based on Aljanabi et al. (1999) with modifications. Solid samples were ground in liquid N_2 and 5.0 mL of samples were added to 4.0 mL of homogenization buffer (200 mM Tris–HCl, 50 mM EDTA, 2.2 M NaCl, 2% CTAB, 0.06% Na_2SO_3, pH 8.0). A detergent solution (2.0 mL of 5% N-lauryl-sarcosine, 2.0 mL of 10% PVP, 2.0 mL of 20% CTAB) was added to the homogenized samples and mixed by inversion for 2–3 min, then incubated for 60 min at 65°C with periodic inversions. After incubation, 10.0 mL of 25:24:1 phenol: chloroform: isoamyl alcohol were added to the samples and mixed by inversion for 2 min. After centrifugation (1,520 *g*, 10 min, 4°C), the supernatant was transferred to a new tube, 10.0 mL of 24:1 chloroform: isoamyl alcohol was added, and mixed by inversion for 2 min. After centrifugation (1,520 *g*, 10 min, 4°C), the supernatant was transferred to fresh tubes, 10.0 mL of isopropanol and 2.0 mL of 6 M NaCl were added, and the tubes were mixed by inversion for 2 min. The samples were then incubated (20°C, 1 h), centrifuged (1,520 *g*, 5 min, 4°C), and the formed pellets were washed two times with 10.0 mL of 70% ethanol. The pellets were dried at room temperature and dissolved in 200 μL of sterile ultrapure water. All DNA samples were quantified in a NanoDrop™ 8000 spectrophotometer (ThermoFisher™). In addition to the total DNA quantification results, all samples from both cultivars (CTC20 and CTC175-A), were also evaluated for the presence of heterologous DNA representing *cry1ab* (GenBank Accession No. AY326434.1) and *nptII* (GenBank Accession No. U00004) genes and the endogenous *ubi1* gene (GenBank Accession No. CA179923.1) genetic elements by TaqMan multiplex analysis (**Table 2**).

The TaqMan® Multiplex Assay protocol for DNA detection was performed as follows: the reactions were performed in multiplex form to amplify simultaneously one of the two combinations: *cry1Ab*/*ubi1* or *nptII*/*ubi1*. PCR was performed in a 96-well optical plate ("MicroAmp® Fast Optical 96-Well Reaction Plate™," Life Technologies). The performance of the assays in each processing sample type (e.g., primary juice, bagasse, etc.) was assessed by adding known quantities of the specific DNA for *cry1ab*, *nptII*, and *ubi1* to processing fractions produced from the CTC20 isoline. All samples were normalized for final DNA concentration of 10 ng/μL and 40 ng were used for each PCR. For samples with DNA concentrations below limit of detection (LOD), 4 μL of DNA solution was used. For positive control samples, two known concentrations of each target gene were used (0.5 and 0.05 ng of DNA).

Reactions were assembled using samples added to a mixture of the following reaction components: 1× Taqman® II Mix Universal Buffer UNG (Applied Biosystems™); forward and reverse primers, each at 500 nM concentration; probes at a final concentration of 200 nM for the multiplex assay cry1Ab/ubi; forward and reverse primer, each at 300 nM concentration; probes at a final concentration of 200 nM for the multiplex assay nptII/ubi; and water in sufficient quantity to make up a final volume of 20 μL. Plates were sealed with optical adhesive film for real-time PCR MicroAmp® (Applied Biosystems™) and PCR was performed in a thermocycler 7500 Fast Real-Time PCR System (Applied Biosystems™) using the following amplification parameters: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. The primers and probes sequences used are detailed in **Table 2**.

Each PCR amplification curve was examined to determine the presence (+) or absence (−) of DNA by comparing with the respective amplification curve of the positive control. The control DNA concentrations (0.5 and 0.05 ng), were chosen because they represent reliable detection limits of the methodology. Samples that had amplification (Cq) values higher than the positive control at the lowest concentration of DNA (0.05 ng), were considered as non-specific amplifications (<LOD).

Detection of Total Proteins in Laboratory Production Fractions

For the extraction and quantification of total proteins, an aliquot (500 μL) of samples taken from the laboratory production of sugar (i.e., leaf, bagasse, juice, filter cake, clarified juice, syrup, molasses, sugar, phlegm, and vinasse) was mixed with 750 μL of protein extraction buffer (0.01 M phosphate buffered saline: 0.138 M NaCl; 0.0027 M KCl, 0.05% TWEEN® 20, pH 7.4), homogenized and centrifuged (10 min, 7.690 *g*). After centrifugation, 600 μL of supernatant was transferred to new tubes and the

TABLE 2 | Primers and probes sequences used to identify exogenous (*cry1Ab* and *nptII*) and endogenous (*ubi*) genes present in CTC175-A by qPCR.

Target	PCR product (pb)	Primer (5′ → 3′)	Primer (5′ → 3′)	Probe
cry1ab	102	GTGGACAGCCTGGACGAGAT	GAAGCCACTGCGGAACATG	CCCCTCAGAACAAC
nptII	103	GCTCACCCTGTTGTTTGGTGTT	AGCCTCTCCACCCAAGCG	CTTCTGCAGGTCGACTC
ubi1	63	ACCATTACCCTGGAGGTTGAGA	GTCCTGGATCTTCGCCTTCA	CTCTGACACCATCGAC

samples were analyzed for total protein concentration using the Bradford method. A seven-point standard curve with concentrations ranging from 125 to 2,000 mg/mL of bovine serum albumin was produced.

A 96-well flat-bottomed plate was assembled using 10 µL of buffer (null control), 10 µL of each standard (in duplicate) and 10 µL of each sample studied. Bradford solution (200 µL) was added (Bio-Rad Protein Assay Dye Reagent Concentrate™) to each well in a 1:4 ratio (dye:water). The plate incubated on the bench for 5 min and was read on a M2-SpectraMax spectrophotometer (Molecular Devices™).

Detection of Cry1Ab and NptII Proteins in Laboratory-Processed Samples

All samples were analyzed for Cry1Ab protein presence using the "QualiPlate™ ELISA Kit for Cry1Ab/Cry1Ac" (ENVIROLOGIX ™) according to the manufacturer's recommendations. Data were generated using the SpectraMax-M2 Spectrophotometer (Molecular Devices™). A known amount of Cry1Ab protein (~ 0.50 ng) was used to spike a portion of the samples obtained during the production of sugar and ethanol from CTC20-derived samples to obtain an expected final concentration of approximately 3.0 ng/mL of Cry1Ab protein for each sample. A serial dilution curve of the Cry1Ab protein was positioned adjacent to investigated samples in the plate to determine the LOD of the assay. The dilution curve ranged from 50 ng to 3.125 ng/mL of protein.

Collection of Conventional Sugar Samples From Brazilian Sugarcane

Samples from the industrial processing of conventional sugarcane were collected at two different types of industrial sugarcane mills in Brazil: a tandem roller type ("mill F") and a diffuser type ("mill C"). Nine sample types were collected from each mill, in the harvest 2016/17: bagasse, primary juice, filter cake, clarified juice, syrup, molasses, vinasse, raw sugar, and flegma. Leaves from CTC20 variety were used as a control sample that does not contain the heterologous DNA and newly expressed proteins. All samples were transported on ice (2–4°C) and stored at −80°C. Leaves from CTC20 variety were used as a control. All samples were stored and transported to CTC on blue ice (2–4°C), and immediately frozen after arriving.

Total and RuBisCO DNA Detection in Processing Fractions Obtained From Brazilian Sugarcane Processing Mills

DNA of each fraction was isolated (5.0 mL wet or dry samples in a 50.0 mL conical tube) following the DNA extraction protocol described by Aljanabi et al. (1999). DNA samples were concentrated in an Eppendorf™ Vacufuge™ to a final volume of 0.2 mL before quantification in a Qubit® fluorometer (LifeTechnologies) following the protocol suggested by the manufacturer. DNA samples were also assessed for quality by visualization on ethidium bromide-stained agarose gels.

The sequence of the RuBisCO large subunit of *Saccharum* hybrid cultivar SP80-3280 (GenBank: AE009947.2) was used to design primers for PCR assays (*Saccharum* hybrid cultivar SP-80-3280 chloroplast, complete genome: 119082-120512). Primers were designed to specifically amplify fragments of different sizes. The sequences of the primers and the expected fragment sizes are given in **Table 3**.

A dilution curve was prepared using total DNA from sugarcane leaves. Decreasing concentrations ranged from 50 to 0.0125 ng. Reactions were prepared in a final volume of 25 µL using the following reaction components: 1× DreamTaq Green PCR Master Mix (Thermo Scientific), 0.2 µM of each primer, and 5 µL of genomic DNA of known concentrations, to have five different points of dilution curve for each tested pair of primers (50, 25, 12.5, 0.125, and 0.0125 ng).

PCRs were performed on the Proflex® thermal cycler (Applied Biosystem), according to the following step-cycle program: initial denaturation step at 92°C for 2 min; 30 cycles consisting of denaturation at 92°C for 30 s, annealing at 60° for 40 s (primers combinations 1, 2, and 3) or annealing at 50°C (primers combinations 4 and 5), and extension at 72°C for 60 s; final extension step at 72°C for 7 min. After the amplification, PCR products were electrophoresed on 2% agarose gels in 1× TBE solution, stained with 0.4 µg/mL ethidium bromide, visualized under ultraviolet light (UV), and registered with transilluminator and software L.PIX Loccus Biotechnology.

Reactions were performed in 25 µL final volume with variable amounts of template. For fractions from which DNA was quantifiable, serial dilutions were prepared; seven points (10, 5, 0.5, 0.25, 0.025, 0.0025, and 0.00125 ng) were amplified in each PCR. When DNA concentrations from fractions were below the LOD (<LOD), an arbitrary volume of sample was added to the amplification reactions. Thus, for raw sugar and flegma from the tandem roller mill samples and, raw sugar, flegma, and clarified juice samples from diffuser mill 5, 2.5, 1.25, and 0.5 µL of template were used. DNA from leaf was used as positive control (2.5 ng). In parallel, an aliquot of the same matrix of each sample was spiked with DNA from sugarcane leaves. Again, a serial dilution was done to have an input of approximately 2, 1, 0.1, 0.01, and 0.001 ng of DNA in each reaction. For raw sugar and flegma from both types of mills and for clarified juice from diffuser mill, the dilution curve consisted of 12.5, 6, 3, and 1 pg were used. Reaction components and the cycling program followed as described previously, using 28 amplification cycles. PCR products were electrophoresed on 2% agarose gels.

Total Protein Detection in Processing Fractions Obtained From Brazilian Sugarcane Processing Mills

After freezing, 6.0 mL of each sample was aliquoted in six tubes (1.0 mL each) and lyophilized for 6 days at −60°C with exception

TABLE 3 | Primers name, primers sequences, and expected size of amplified fragments.

Combinations	Primer name	Sequence 5′ → 3′	bp
1	SoRcbL_TqM.F	CGCCTCACGGTATCCAAGTT	246
	SoRcbL_R.1	CGGTTTCGGCTTGTGCTT	
2	SoRcbL_TqM.F	CGCCTCACGGTATCCAAGTT	437
	SoRcbL_R.2	TGCTCGGTGAATGTGAAGAAG	
3	SoRcbL_F	CGGAGTACGAAACCAAGGATAC	809
	SoRcbL_R.2	TGCTCGGTGAATGTGAAGAAG	

of flegma. Samples from leaves and bagasse were further ground to a homogeneous powder and protein extraction was performed as described by Cullis et al. (2014) with minor modifications. Lyophilized samples from individual tubes combined into a 15-mL polypropylene tube and prepared as solutions, where former solid samples (leaves, bagasse, and filter cake) were dissolved at a 3% (w/v) in water. Similarly, primary juice, clarified juice, syrup, molasses, raw sugar, and vinasse were dissolved in a 10% (w/v) in water. Flegma was prepared as a solution 20% (v/v) in water. This procedure was done in duplicate, with half of the mill's fractions (controls) spiked with 1,000 ng of total protein, previously extracted from sugarcane leaves and quantified using microplate "Micro BCA protein assay" (ThermoFisher). In the case of raw sugar and flegma, aliquots were spiked with 10 µg of total protein. All solutions were adjusted to 1% sodium dodecyl sulfate (SDS) + 10 mM dithiothreitol + 10 mM Tris–HCl pH 7.5 + 0.5 mM PMSF (SDS extraction buffer) and placed at 65°C for 60 min with occasional mixing by inversion. Tubes were centrifuged (6,500 *g*) for 15 min at room temperature. To 2 mL of the supernatant, 3 mL of 1% sodium deoxycholate were added followed by 1.25 mL of 50% trichloroacetic acid. After mixing and incubating on ice for 15 min, the tubes were centrifuged at 6,500 *g* for 15 min at 4°C, supernatant was discarded, and the pellet drained for 5 min. Pellets were then washed with 1.5 mL of acetone by vigorous mixing for 15 s followed by incubation at 25°C for 15 min with occasional mixing. Samples were placed on ice for 10 min, centrifuged, the supernatants removed, and the tubes drained at room temperature. Next, 1.5 mL of 85% acetone was added with mixing, and the tubes were centrifuged, drained, and dried at 37°C for 15 min. The precipitate was dissolved in 0.5 mL of 0.5% SDS + 10 mM Tris–HCl pH 7.5 at 65°C for 20 min, with occasional mixing. Assuming 100% of recovery, the concentration of total proteins in spiked samples per microliter of resuspended extract, should be 2 ng/µL. Protein content was determined in both, original mill fractions and spiked mill fractions (controls), using the microplate Micro BCA protein assay (ThermoFisher) as recommended by the manufacturer. SDS-PAGE was used to check extracted protein quality of each sample. About 2–20 µg of total protein were diluted in sample buffer (2× Laemmli Buffer, Biorad, USA) and denatured at 100°C for 5 min. Proteins were separated under denaturing conditions on a 4–20% polyacrylamide gel (Mini-PROTEAN TGX, Biorad, USA) ready to use. At the end, protein gels were stained with Coomassie Brilliant Blue (EZBlue, Sigma, USA).

RuBisCO Protein Detection

ELISA was performed according the manufacturer's recommendations ("Plant RuBisCO ELISA Kit"—Catalog # MBS705973—MyBioSource). The detection range described in the kit's protocol is 3.12–800.0 µg/mL. Thus, using the standard sample solution supplied with the commercial kit a standard curve was prepared with five points of known RuBisCO concentration (µg/mL). The protein sample eluent (0.5% SDS + 10 mM Tris–HCl) serves as the zero standard and the curve blank. The total protein samples and the buffer used to elute the protein after extraction were diluted to 1/2, 1/5, 1/10, and 1/100. The mean absorbance of each buffer dilutions was used as a blank for each sample dilution. The concentration read from the standard curve was multiplied by the dilution factor. The optical density of each well was determined using a microplate reader (SpectraMax, Molecular Devices, USA) set to 450 nm with wavelength correction of 540 and 570 nm.

RESULTS AND DISCUSSION

Expression of Cry1Ab and NptII on Tissues of CTC175-A Events

The construct used to obtain CTC175-A event was designed to express Cry1Ab preferentially in leaves, where the sugarcane borer lays its eggs and starts its development. The promoter used to drive Cry1Ab expression, PEPC, is known to confer preferential expression in photosynthesizing tissues (Harrison et al., 2011). Therefore, as expected, the highest concentrations of Cry1Ab were found in leaf tissue in all evaluated sites; much lower levels were found in roots and stalks [below the limit of quantification (LOQ) of ≤235 ng/g FW tissue] (**Table 4**).

In **Table 4**, Cry1Ab expression values in leaves were expressed as mean of four repeats, with their respective SE and SDs, for each site. As for root and stalk tissues, expression of Cry1Ab is much lower and some repeats data were below of LOQ of ELISA assay. When at least one repeat had measurement above LOQ, data of Cry1Ab expression for those repeats above LOQ were reported directly without any statistical analysis. It was only possible to detect Cry1Ab expression in stalks, the raw material for sugar and ethanol production, for only one repeat of each site, Conchal (310 ng/g FW tissue) and Paranavaí (370 ng/g FW tissue).

The expression of NptII on event CTC175-A was solely required for selecting transformed events during the transformation

TABLE 4 | Concentration of Cry1Ab protein in tissues of CTC175-A event evaluated *via* ELISA ($n = 4$).

Site	Tissue	Cry1Ab (µg/g FW)	SD	SE
Jaboticabal	Stalk	<LOQ	–	–
	Leaf	64.01	9.59	4.8
	Root	0.40/0.55/0.58[a]	–	–
Montividiu	Stalk	<LOQ	–	–
	Leaf	40.7	10.5	5.2
	Root	0.81/0.83/0.87[a]	–	–
Piracicaba	Stalk	<LOQ	–	–
	Leaf	64.9	11	5.5
	Root	<LOQ	–	–
Conchal	Stalk	0.31[b]	–	–
	Leaf	63.4	6.7	3.4
	Root	0.67/0.70[c]	–	–
Uberlândia	Stalk	<LOQ	–	–
	Leaf	67.9	20.6	10.3
	Root	<LOQ	–	–
Paranavaí	Stalk	0.37[b]	–	–
	Leaf	29.6	3.1	1.6
	Root	0.84[b]	–	–

Limit of quantification for leaf, stalk, and root tissues: LOQ ≤ 0.235 µg/g.
[a]*Three repeats above LOQ.*
[b]*One repeat above LOQ.*
[c]*Two repeats above LOQ.*

process. The *Ubi* promoter driving expression of the *nptII* gene in the transformation cassette is known to be expressed in rapidly dividing tissues (Christensen et al., 1992). Therefore, results show low levels of NptII expression in leaves 0.07–0.16 μg/g FW and even lower expression in roots ranging from below the LOQ (<34 ng/g FW) to 70 ng/g FW. NptII expression was at or below detectable levels in sugarcane stalks (<34 ng/g FW) (**Table 5**).

At **Table 5**, as for Cry1Ab, NptII expression values in leaves were expressed as mean of four repeats, with their respective SEs and SDs, for each site. As for root and stalk tissues, expression of NptII is much lower and some repeats data were below LOQ. When at least one repeat had measurement above LOQ, it was decided to report data of NptII expression for those repeats above LOQ, without any statistical analysis.

These results indicate that sugarcane stalks, which are the raw material for sugar and ethanol production, presents originally low levels of heterologous protein expression. This is not surprising due to the nature of promoters used to drive gene expressions and the fact that sugarcane naturally presents negligible protein levels in stalks (OECD, 2011). In fact, the search for promoters that ensures high protein expression levels in sugarcane stalks is still a scientific challenge (Damaj et al., 2010).

Composition of Sugar Obtained From the Laboratory Processing

The quality of the harvested sugarcane for industrial processing is an important consideration for processing mills because stalk quality directly affects the sugar and ethanol production potential (Garcia, 2012; Santos et al., 2012; Santos and Borem, 2013). One batch of sugarcane juice for CTC175-A event and one for CTC20 cultivar were prepared, therefore, the values presented at **Tables 6** and **7** should be evaluated as single measures and not as estimates of quality parameters of juices and sugars produced from CTC175-A and CTC20. Despite this, the results of quality parameters of both juices were within the recommended range for these sugarcane analytes (**Table 6**), confirming that the raw material used for the laboratory production for sugar and ethanol in this study was acceptable. This procedure is commonly used by Brazilian mills to evaluate and to pay according to the content of sucrose (Pol% juice) in sugarcane (Bruijn, 1998). The other parameters were evaluated for key sugarcane processing steps. Overall, juices produced in laboratory scale resembled juice ordinally processed in Brazilian mills.

In Brazil, it is the usual practice to employ COPERSUCAR specifications to classify sugars for different industrial applications. According to the physicochemical parameters evaluated (**Table 7**), sugar produced from both the event CTC175-A and CTC20 conventional were classified as "Type 3C" according to the classifying parameters: conductometric ashes ≤ 0.1%, color ICUMSA ≤ 400 and sugar content (Pol Z) above 99.5%, published by COPERSUCAR (2015). The high quality of Type 3C sugar produced, which technically can be labeled as "white sugar," is not surprising even though the sugar production method employed here was the typical method for production "raw sugar." This higher grade result can also be obtained in real world sugar production when the quality of starting sugarcane juice is high (Santos et al., 2012).

TABLE 5 | Concentration of NptII protein in tissues of CTC175-A event evaluated *via* ELISA ($n = 4$).

Site	Tissue	NptII (μg/g FW)	SD	SE
Jaboticabal	Stalk	<LOQ	–	–
	Leaf	0.15	0.02	0.01
	Root	<LOQ	–	–
Montividiu	Stalk	0.02[a]	–	–
	Leaf	0.08	0.02	0.01
	Root	0.04/0.04[b]	–	–
Piracicaba	Stalk	0.01[a]	–	–
	Leaf	0.16	0.02	0.01
	Root	0.07	0.01	0.01
Conchal	Stalk	0.02[a]	–	–
	Leaf	0.13	0.03	0.02
	Root	0.05	0.01	0.01
Uberlândia	Stalk	0.01[a]	–	–
	Leaf	0.03	0.07	0.03
	Root	0.04[a]	–	–
Paranavaí	Stalk	0.02/0.01[b]	–	–
	Leaf	0.13	0.05	0.02
	Root	0.06/0.24[b]	–	–

Limit of quantification for leaf tissues: LOQ ≤ 0.034 μg/g. Limit of quantification for stalk and root tissues: LOQ ≤ 0.0094 μg/g.

[a]*One repeat above LOQ.*

[b]*Two repeats above LOQ.*

TABLE 6 | Results of sugarcane quality analysis.

Characteristic	Unit	Recommended values (Santos and Borem, 2013)	CTC20	CTC175-A
Fiber	% sugarcane	10–13	10.72	10.41
Starch	mg/kg	<1,000	875	948
Brix	% juice	>14	21.02	20.84
Dextran	mg/kg	<10	<10	<10
RS	% juice	<0.8	0.47	0.47
TRS	% juice	>15	20.54	20
pH	–	4.8–5.8	5.4	5.3
Pol	% juice	>14	19.43	19.26
Purity	% juice	>85	92.44	92.42

Single batch results (n = 1).

TABLE 7 | Physicochemical parameters of example raw sugar lots produced from control cultivar CTC20 and CTC175-A including relevant copersucar raw sugar classification specifications.

Characteristic	Unit	CTC20	CTC175-A	Type 3C
Starch	mg/kg	294	207	–
Conductometric ash	% m/m	0.02	0.03	max 0.1
ICUMSA color (MOPS)	IU	200	246	max 400
Dextran	mg/kg	<10	<10	–
Filterability	mL–min	45–5	43–5	–
Acid beverage floc	–	Negative	Negative	–
Alcohol floc	Abs	0.055	0.083	–
RS	% m/m	<0.06	<0.06	–
Polarization	Z	99.74	99.67	min 99.5
Turbidity	NTU	15	40	–

Single batch results (n = 1).

Detection of *cry1Ab* and *nptII* DNA Sequences of Fractions of Laboratory Processing of CTC175-A

The results of detection of gene sequences of DNA (*cry1Ab*, *nptII*, and endogenous *ubi*) in samples from the laboratory fractions of sugarcane processing showed the presence of endogenous DNA (*ubi* gene) in leaf, bagasse, primary juice, and in the precipitated fraction of clarification process filter cake, both from the laboratory processing of event CTC175-A and CTC20 (**Tables 8** and **9**). The molasses fractions from CTC20 but not from CTC175-A event also showed the presence of the *ubi* gene (**Tables 8** and **9**). The vinasse sample showed detection of *ubi* gene at levels equivalent to presence of a DNA concentration below 0.05 ng (positive control) (**Table 8**) and for the juice processed from CTC175-A (**Table 9**). Flegma also showed detection of *ubi* gene at levels equivalent to presence of a DNA concentration below 0.05 ng (positive control) (**Table 9**).

The results for amplification of *cry1Ab* gene from CTC175-A event were completely concordant with the results of *ubi* amplification in CTC175-A and CTC20, revealing positive amplifications for the less processed fractions (leaf, bagasse, and primary juice) and the precipitated residue filter cake (**Table 8**). Additionally, vinasse from CTC175-A also showed detection of *cry1Ab* gene at levels equivalent to presence of a DNA concentration below 0.05 ng, as for *ubi* gene from CTC175-A and CTC20. All samples spiked with 0.05 ng of DNA from *cry1Ab* gene showed expected amplifications, ensuring the absence of matrix negative influence on DNA amplifications, at least at the level of quantification of this assay. These results clearly show that the *cry1Ab* DNA was degraded and/or removed in the juice clarification step, and subsequent downstream fractions, including raw sugar, did not contain detectable levels of *cry1Ab* gene DNA.

The results of detection of *nptII* gene are similar to those for *cry1Ab* detection (**Table 9**). There was DNA detection in all processing samples spiked with appropriated amount to detect 0.05 ng of *nptII* DNA (positive controls) showing that the gene, if present could be amplified and detected in each fraction. Samples obtained from byproducts of CTC20 processing detected DNA presence only for the *ubi* gene in unprocessed samples (leaves) or less processed (bagasse, juice, and filter cake), except for molasses which also showed amplification. The flegma sample showed detection equivalent to a DNA concentration below 0.05 ng (**Table 9**). Samples from of event CTC175-A event showed DNA presence for both the genes (*ubi* and *nptII*) in samples without processing (leaf) or minimal processing (bagasse, juice, and filter cake) as described for detecting the endogenous gene in the CTC20 cultivar (**Table 9**).

These results obtained by TaqMan assay are consistent with the findings of Cullis et al. (2014) who also found a dramatic reduction in total sugarcane DNA quantity upon production of the clarified juice, the common starting material for production of raw sugar and ethanol production. No heterologous DNA was detected by PCR amplification in the final products raw sugar or flegma (the starting material for ethanol production).

Detection of Total Proteins in Fractions of Laboratory Production of Sugar and Ethanol

Protein quantification methodology was effective in detecting measurable amounts of protein (above LOD) for samples of leaves, bagasse, and primary juice from event CTC175-A and the CTC20 conventional (**Table 10**) collected in the laboratory-scale preparation of sugar and ethanol. The values presented at **Table 10** should be evaluated as single measures and not as estimates of total protein content in fractions of processing of CTC175-A and CTC20 because there was only one preparation for each material. It was not possible to detect measurable total proteins in samples after juice clarification for both, CTC175-A

TABLE 8 | Results of gene amplification of *ubi* (endogenous gene) and *cry1Ab* in samples collected during the laboratory sugarcane processing to produce sugar and ethanol from CTC20 and CTC175-A.

	CTC20 + 0.05 ng DNA (*cry1ab*)		CTC20		CTC175-A	
	ubi	*cry1ab*	*ubi*	*cry1ab*	*ubi*	*cry1ab*
Leaf	+	+	+	–	+	+
Bagasse	+	+	+	–	+	+
Primary juice	+	+	+	–	+	+
Filter cake	+	+	+	–	+	+
Clarified juice	+	+	–	–	–	–
Syrup	+	+	–	–	–	–
Molasses	+	+	+	–	–	–
Sugar	+	+	–	–	–	–
Flegma	+	+	–	–	–	–
Vinasse	+	+	<LOD	–	<LOD	<LOD
Evaporation water	+	+	–	–	–	–
Condensation water	+	+	–	–	–	–

Samples obtained from cultivar CTC20 were intentionally spiked with 0.5 and 0.05 ng of DNA from CTC175-A event. +: presence; –: absence; <LOD: the amplification curve shows cq later than the same sample at a concentration of 0.05 ng of total DNA per reaction.

TABLE 9 | Results of gene amplification of *ubi* (endogenous gene) and *nptII* in samples collected during the sugarcane laboratory processing to produce sugar and ethanol from CTC20 and CTC175-A.

	CTC20 + 0.05 ng DNA (*nptII*)		CTC20		CTC175-A	
	ubi	*nptII*	*ubi*	*nptII*	*ubi*	*nptII*
Leaf	+	+	+	–	+	+
Bagasse	+	+	+	–	+	+
Primary juice	+	+	+	–	+	+
Filter cake	+	+	+	–	+	+
Clarified juice	+	+	–	–	<LOD	<LOD
Syrup	+	+	–	–	–	–
Molasses	+	+	+	–	–	–
Sugar	+	+	–	–	–	–
Flegma	+	+	< LOD	–	<LOD	–
Vinasse	+	+	–	–	+	–
Evaporation water	+	+	–	–	–	–
Condensation water	+	+	–	–	–	–

Samples obtained from cultivar CTC20 were intentionally spiked with 0.5 and 0.05 ng of DNA from CTC175-A event. +: presence; –: absence; <LOD: the amplification curve shows cq later than the same sample at a concentration of 0.05 ng of total DNA per reaction.

TABLE 10 | Total protein quantification after Bradford extraction in fractions of laboratory sugarcane processing.

Sample	Total protein (mg/mL)	
	CTC175-A	CTC20
Leaf	0.64	0.79
Bagasse	0.05	0.02
Primary juice	0.06	0.12
Clarified juice	Not detected	Not detected
Filter cake	Not detected	Not detected
Syrup	Not detected	Not detected
Molasses	Not detected	Not detected
Sugar	Not detected	Not detected
Phlegma	Not detected	Not detected
Vinasse	Not detected	Not detected
Evaporation water	Not detected	Not detected
Condensation water	Not detected	Not detected

Single batch results (n = 1).

and CTC20, indicating this process leads to either protein degradation or precipitation.

Detection of Cry1Ab in Raw Sugar Produced in Laboratory Scale

Cry1Ab protein was detected in leaves, bagasse, and primary juice produced in laboratory from event CTC175-A sugarcane. The remaining samples that have undergone various chemical and/or heat treatments during the manufacturing process of obtaining sugar and alcohol, as well as the final processed products (sugar and flegma) showed no detectable Cry1Ab protein (**Table 11**). As expected, samples from CTC20 cultivar did not contain detectable Cry1Ab protein whereas samples from CTC20 spiked with Cry1Ab protein showed detection of protein in all cases, demonstrating lack of matrix interference with the detection assay. Molasses and vinasse samples were reported as <LOD because, although there was antibody reaction for these samples, the OD reading was below the lowest point of the dilution curve (3.125 ng).

Detection of DNA, Total Proteins, and RuBisCo in Fractions of Industrial Processing

The SDS-PAGE evaluation from samples from diffuser mill revealed a protein smear in all samples (Figure S1A in Supplementary Material). It was possible to detect smeared proteins in samples of leaves, primary juice and filter cake. It was not possible to detect total proteins from clarified juice and downstream samples (syrup, molasses, and raw sugar) (Figure S1A in Supplementary Material). It was not possible to detect proteins in raw sugar samples (Figure S1B in Supplementary Material) produced in tandem roller and diffuser mills.

The evaluation of total DNA from samples from the Brazilian mill processing fractions revealed that the quantity of extracted total DNA ranged from 173 ng/μL in bagasse to 1.36 ng/μL in clarified juice from samples derived from the tandem roller mill (**Table 12**). Raw sugar and flegma were below the LOD of Qubit® Quantitation Assay Kit (0.2 ng). Samples from the diffuser mill presented as much as 16.6 ng of DNA per μL of bagasse to approximately 1 ng/μL in the molasses fraction. Samples from raw sugar, flegma, clarified juice, and syrup were below of LOD of Qubit® reagents (**Table 12**). Samples from sugarcane leaves yielded as much as 880 ng/μL of DNA.

TABLE 11 | Presence (+) and absence (–) of Cry1ab protein in fractions samples from the industrial processing of CTC20 and CTC 175-A Varieties, using ELISA methodology.

Samples	CTC175-A	CTC20	CTC20 + Cry1ab
Leaf	+	–	+
Bagasse	+	–	+
Primary juice	+	–	+
Clarified juice	–	–	+
Filter cake	–	–	+
Syrup	–	–	+
Molasses	–	–	<LOD
Sugar	–	–	+
Phlegma	–	–	+
Vinasse	–	–	<LOD
Evaporation water	–	–	+
Condensation water	–	–	+

<LOD: below protein detection level per the dilution curve of known concentrations.

TABLE 12 | DNA quantification for each processing fraction sample from two types of mills.

DNA quantification		
Sample	**Tandem roller mill (ng/μL)**	**Diffuser mill (ng/μL)**
Leaves CTC20	880.0	
Bagasse	173	16.6
Primary juice	11.5	6.4
Clarified juice	1.36	<LOD
Filter cake	26.8	16.5
Syrup	1.84	<LOD
Molasses	2.69	0.93
Vinasse	37.2	26.8
Raw sugar	<LOD	<LOD
Flegma	<LOD	<LOD

Single batch results (n = 1).

TABLE 13 | RuBisCO DNA detection in fractions collected during industrial processing of sugar and ethanol production from two types of sugarcane mills.

	Diffuser mill		Tandem roller mill	
	Fraction samples	Fraction samples spiked (+)	Fraction samples	Fraction samples spiked (+)
Bagasse	+	+	+	+
Primary juice	+	+	+	+
Filter cake	+	+	+	+
Clarified juice	<LOD	[a]	+	+
Raw sugar	<LOD	[a]	<LOD	[a]
Flegma	<LOD	[a]	<LOD	[a]
Vinasse	+	[b]	+	[b]
Leaf	+		+	

+: positive detection; <LOD: below of limit of detection; spiked (+): samples contaminated with DNA detection of 0.01 ng (nanogram).

[a]*Positive amplification for samples contaminated with DNA 3 pg of DNA.*

[b]*Positive amplification for samples contaminated with DNA 100 pg of DNA.*

The values presented at **Table 12** should be evaluated as single point estimates of DNA content in fractions from tandem roller and diffuser Brazilian mills. Overall, samples from both mills showed a trend of decreasing DNA concentration throughout the processing steps. The final products of processing (raw sugar and flegma) did not presented DNA above the LOD of this assay for both types of mills (**Table 12**).

Primer combinations 1 and 2 (**Table 3**) were used to obtain results of RuBisCo DNA detection with samples from both types of mills. The results from diffuser mill showed RuBisCo DNA detection for all samples except clarified juice, raw sugar, and flegma. The positive controls (i.e., spiked samples) yielded amplification down to the 0.01 ng level for all samples except vinasse, which could only be observed at the 0.1 ng level. Spiked samples of clarified sugar, raw sugar and flegma supported amplification at 3 pg of DNA. The results from tandem roller mill fractions were similar with those found from diffuser mill samples and indicates that RuBisCo DNA can be detected in all processing fractions except raw sugar and flegma (**Table 13**). The positive controls (i.e., spiked samples) yielded amplification down to the 0.01 ng level for all samples except vinasse, which could only be observed at the 0.1 ng level. Spiked samples of raw sugar and flegma supported amplification at 3 pg of DNA. Therefore, these results are concordant in not identifying RuBisCo DNA in raw sugar and flegma.

TABLE 14 | Total protein quantification using BCA in fraction collected from two types of mills.

Sample	Diffuser mill total protein (µg/mL)	Tandem roller mill total protein (µg/mL)
Leaf	~1.500	
Bagasse	133	267
Primary juice	1.319	1.237
Filter cake	623	933
Clarified juice	13	54
Syrup	8	24
Molasse	88	418
Raw sugar	4	10
Flegma	1	9
Vinasse	581	284

Single batch results (n = 1).

The values obtained for Total protein and RuBisCo quantification in processing fractions of tandem roller and diffuser Brazilian mills should be evaluated as single point estimates. In samples collected from mills, BCA protein quantification demonstrated that most of protein content present at sugarcane juice is eliminated in the precipitated filter cake (**Table 14**) resulting in a protein content in clarified juice at least two orders of magnitude lower than in primary juice. The final protein content in raw sugar and in flegma is minimal (**Table 14**).

Results of RuBisCo quantification were concordant with evaluation of BCA total protein quantification. **Figure 3** shows the results for ELISA assay for RuBisCO concentrations in samples

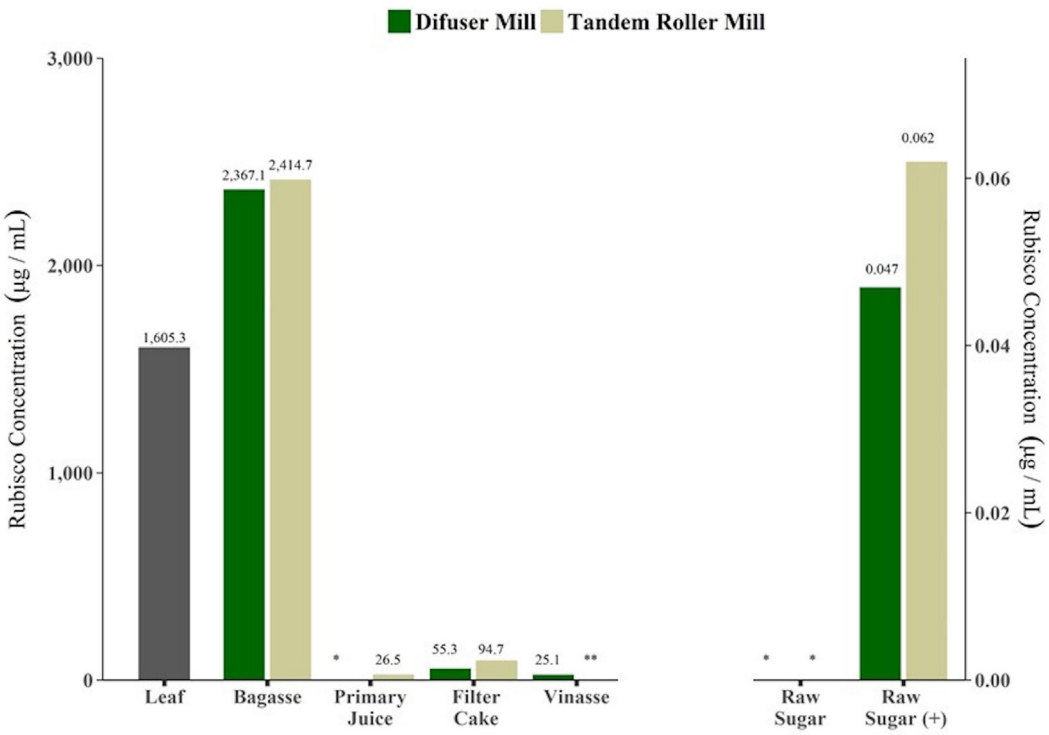

FIGURE 3 | Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) protein detection in by product samples collected during industrial process for sugar and alcohol production, from two types of sugarcane mills, by ELISA assay. Single batch results (*n* = 1). *: below limit of detection (LOD). **: not analyzed. Raw sugar (+): samples of raw sugar spiked with 10 µg of total protein before extraction.

from fractions obtained from commercial tandem and diffuser mills processing plants. Leaf and bagasse are far above the range of quantification (3.12–800 µg/mL), therefore are not accurate estimates. RuBisCO protein was also detected in samples of primary juice, filter cake and vinasse. In the case of primary juice, it was not possible to quantify RuBisCO in samples from diffuser mill. Samples of vinasse from tandem roller mill were not analyzed. As expected, the concentrations of RuBisCO were below the LOQ in samples of raw sugar produced from both type of mills. In samples of raw sugar spiked with 10 µg of RuBisCO protein before extraction, ELISA was sensitive enough to detect as little as 0.05 or 0.06 µg/mL of RuBisCO, confirming that the protein could have been detected at those levels if it was present in raw sugar.

CONCLUSION

The results presented in this study demonstrates that event CTC175-A presents very low expression of Cry1Ab and nptII proteins in stalks, the raw material for sugar and ethanol production. This result is in agreement with the design of the DNA cassette used to obtain this event, that was constructed to drive high levels of Cry1Ab in leaves. Besides, several assays of fractions of laboratory processing strongly suggests that total DNA, total protein, heterologous DNA and Cry1Ab protein are degraded during processing, leading to concentrations that are not easily detected by commonly used methodology employed to evaluate the presence of GMOs or GMOs derivative in food/feed.

Three lines of evidence clearly establish that raw sugar does not contain detectable levels of either the inserted heterologous DNA or expressed proteins. First, published studies of total DNA and protein loss during stalk processing to refined sugar showed levels of <1 pg total DNA/g refined sugar and ~1 µg total protein/g refined sugar (Cullis et al., 2014). Given these extremely low detection levels for total DNA and protein, it is expected that the small quantities of heterologous DNA and newly expressed protein would also be no detectable. Second, studies presented herein (**Tables 11–14**; **Figure 3**), tracked the concentrations of total protein and RuBisCO protein during stalk processing to refined sugar in two types of commercial processing plants in Brazil. RuBisCO is the single most abundant stalk protein (up to 30% of total plant protein) with very high DNA copy number. These studies confirmed the results of Cullis et al. (2014) that the extent of protein loss during processing is at least three to four orders of magnitude (2–5 mg of total protein per gram of cane preextraction and 0.75–1.875 µg total protein in raw sugar derived from 1 g of cane). Finally, and most importantly, studies with new event CTC175-A sugarcane stalks, clarified juice, molasses, and raw sugar showed no detectable levels of Cry1Ab protein (by ELISA, <235 ng/g FW tissue) in stalks or processed fractions. Similarly, no heterologous DNA was detected in clarified juice and downstream products including raw sugar. These results are in agreement with the results of other studies that investigated the degradation of specific DNA fragments inserted into genetically modified sugarcane (NptII) or glyphosate-resistant sugar beet (CP4 EPSPS) that reported the complete elimination of the inserted DNA during processing to refined sugar (Klein et al., 1998; Oguchi et al., 2009; Joyce et al., 2013).

In conclusion, results reported here demonstrate lack of detectable protein and DNA from CTC175-A at reasonable levels of sensitivity in processing fractions of sugarcane, including raw sugar, and are in alignment with previous studies reported in Cullis et al. (2014) and Joyce et al. (2013) on sugarcane. Detectability and quantification of these analytes (proteins in particular) are directly relevant to the globally accepted comprehensive safety assessment strategy on biotechnology-derived crops. Quantification forms the underpinning for the exposure component of the risked-based safety assessment; low/no exposure to the heterologous proteins expressed in CTC175-A in conjunction with the extensively reviewed hazard assessment data on those proteins showing no measurable toxicity to humans, animals, or the environment, support the safety conclusions on CTC175-A. Currently, there are no regulations specific to sugarcane related to DNA or protein detection; this work seeks to establish viable parameters to determine levels of exposure to potential toxicants. It is though publications such as this that government and industry standards can be derived and justified.

ETHICS STATEMENT

All manipulation of genetically modified organisms and their derivatives were strictly performed according to Brazilian Biosafety Law 11.105 and CTNBio regulations and required approvals.

AUTHOR CONTRIBUTIONS

ACG, RL, and WO conceived the study; AG, GM, MS, and TF performed experiments; ACG, RL, AG, and MS analyzed the data; ACG, AG, GM, RL, and WO wrote the manuscript; DO, GM, and MS provided supportive information.

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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.2018.00024/full#supplementary-material.

FIGURE S1 | SDS-PAGE gel of protein in fractions of Brazilian mills. M, molecular 838 Marker; BG, bagasse; PJ, primary Juice; FC, Filter Cake; CJ, Clarified juice; SY, syrup; MO, 839 molasses; X, empty lane, L, leaf; RW, Raw sugar. Number following letters indicates spiking of 840 correspondent amount (in µg) of total total protein before protein extraction.

TABLE S1 | Supplemental methodology.

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Capacities for the Risk Assessment of GMOs: Challenges to Build Sustainable Systems

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The need for functional risk assessment bodies in general, and in the biosafety field in particular, demands continued efforts and commitment from regulatory agencies, if results that are sustainable in time are to be achieved. The lack of formal processes that ensure continuity in the application of state of the art scientific criteria, the high rotation in some cases or the lack of experienced professionals, in others, is a challenge to be addressed. Capacity building initiatives with different approaches and degrees of success have been implemented in many countries over the years, supported by diverse governmental and non-governmental organizations. This document summarizes some capacity building experiences in developing countries and concludes that risk assessors taking ownership and regulatory authorities fully committed to developing and retaining highly qualified bodies are a *sine qua non* to achieve sustainable systems. To this end, it is essential to implement "in-house" continuing education mechanisms supported by external experts and organizations, and inter-institutional cooperation. It has to be noted that these recommendations could only be realized if policy makers understand and appreciate the value of professional, independent regulatory bodies.

Keywords: risk assessment, capacity building, biosafety regulatory systems, problem formulation, collective action

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INTRODUCTION

Investments in the establishment of functional biosafety regulatory systems and in periodical revisions for the continued improvement and modernization of existing ones are necessary if the benefits of agricultural biotechnology are to be realized.

Transparent and efficient regulatory systems with clearly defined criteria and procedures to process product applications and that can make timely and predictable science based decisions is a precondition for sustainable investments in research and development, technology transfer and product deployment.

The regulation of genetically modified (GM) crops has been criticized as a constraint to innovation in agriculture, particularly by public sector developers, largely because of the high costs of generating the data required globally by regulatory authorities for commercial approvals. In some countries, this is further complicated by inadequate local capacities to monitor compliance with biosafety measures through timely inspections, guidance and advice. In spite of these criticisms, it is agreed that predictable, consistent regulatory systems can be a powerful stimulus for investments in agricultural innovation.

Capacity building is a critical factor for the development and implementation of functional biosafety regulatory systems and requires a sustained commitment of human and material resources.

Numerous initiatives with different approaches have been implemented in many countries for over 20 years with varied degrees of success, supported by diverse governmental and non-governmental organizations. These programs were primarily aimed to build in-country capacities but also enabled the active participation of country experts in international fora. In fact, inclusive discussions at the regional and international levels are critical to develop consensus on scientific criteria, conceptual tools and common standards for evidence based risk assessment and regulations, ultimately facilitating greater harmonization among countries and regions (OECD, 2005; Bartholomaeus et al., 2015).

A shortcoming of many of these capacity building programs, however, is that while they do help with the understanding of the basic scientific criteria and internationally accepted approaches to risk assessment, the analysis of reference documents and the practical use of these tools to case studies, they do not provide the continued support needed to establish, adopt and then implement these systems in country over time.

Risk assessment is a dynamic, scientific exercise that requires significant technical capacity. The problem formulation methodology is currently considered the starting point, at which the appropriate characterization of the problem is made. The identification of available sources of information and the need for additional evidence to respond to risk hypotheses, subsequently help characterize the risk involved and make a decision about its acceptability and eventually propose risk management or mitigation measures.

In most countries there are not formal specialization options, therefore, only practice and experience make professional risk assessors and this is a lengthy process that may take 3 to 5 years. The lack of "in-house" formal processes to train and update risk assessors on the evolving scientific criteria, the high rotation in some cases, or the lack of experienced professionals and resources in others, can be challenging.

This is especially true in developing countries, but is also a challenge for some developed, mature systems, as discussed in a recent reflection paper for the European Food Safety Authority (EFSA) case (Deluyker, 2017). The author points out to the difficulties to ensure the continued availability of qualified experts for the Scientific Panels (suggests to extend the appointment period of panel members to 5 years instead of the current 3-year term and to be renewable for an extra term). According to the author, another challenge for EFSA is "how to ensure that the EU maintains adequate future expert capacity for scientific assessments. This requires on the one hand that training is offered and on the other that there are adequate opportunities to gain experience."

Workshops, symposia, courses and conferences can be informative and are valuable to raise awareness or catalyze discussions that may aid in the development of strategic programs. However, only the continued engagement with the practice of risk assessment of those who are directly tasked with the responsibility of regulatory oversight, leads to the formation of professional, expert bodies.

The purpose of this paper is to discuss some experiences on capacity building efforts to support regulatory systems in different countries, in order to learn from the past and bring up some ideas for the development of self-sustainable systems in the future. These experiences were shared at a special panel during the 14th ISBGMO meeting that took place in Guadalajara (Mexico) in June, 2017.

A recent, 3 year collaborative program implemented in Paraguay will be discussed in detail regarding the outcomes and the challenges faced during and after the process, as a leading case with common features with several other cases shared at the session. Additional contributions by panel members will be also summarized as examples of capacity building experiences in their respective countries.

Recommendations resulting from this session and similar ones that can follow may contribute to improve and make capacity building programs more efficient and self-sustainable.

PARTNERSHIP FOR BIOSAFETY RISK ASSESSMENT AND REGULATION: A COLLECTIVE ACTION IN PARAGUAY

Activities with agricultural biotechnology were first regulated in Paraguay in 1997, followed by other legal instruments. The more recent, a Decree from 2012, created the National Agricultural and Forestry Biosafety Commission (CONBIO) with the mission to assess, analyze and issue recommendations on all matters related to the introduction, field trials, pre-commercial and commercial release, and other intended uses of GM crops (Ministry of Agricultures and Livestock, 2017). CONBIO identified the need to update the existing framework, so that it would keep up with the evolution of scientific knowledge and experience with genetically engineered technologies.

As part of this process, the Paraguayan Ministry of Agriculture joined the "Partnership for Biosafety Risk Assessment and Regulation" through the signature of a Memorandum of Understanding between the National Agricultural and Forestry Commission and the International Life Science Institute (ILSI) Research Foundation, in November 2012.

The purpose of the Program was to contribute to the improvement of technical capacity for biosafety risk assessment and regulation, so as to further strengthen institutional governance of agriculture biotechnology in Paraguay. Activities were framed within the Partnership for Biosafety Risk Assessment and Regulation, a global project led by ILSI Research Foundation and funded by the World Bank. The purpose of this partnership was *to strengthen the technical capacity of developing country stakeholders in regards to biosafety risk assessment and regulation* (ILSI Research Foundation, 2015).

Importantly, the program plan for Paraguay was designed in response to feedback received in previous meetings with the Paraguayan government representatives to assess current capacities and identified needs, as well as with inputs received in interviews with researchers, regulators, farmers and other

stakeholders interested in agricultural biotechnology. A close follow-up along the implementation phase of the program, allowed incorporating suggestions and recommendations from participants so adjustments could be made accordingly.

One of the factors that contributed to this program's success was the collective action, as researchers, regulators, professionals and technicians from the CONBIO, ILSI Research Foundation and ILSI Argentina worked closely and fruitfully in the implementation and follow-up of the program. The program also benefited from the active collaboration of other organizations such as the National University of Asunción and the Argentine Council for Information and Development of Biotechnology (Argenbio), IICA's office in Paraguay (Interamerican Institute for Agricultural Cooperation) and the Institute of Agricultural Biotechnology in Paraguay (INBIO).

Broadly, activities included seminars and workshops on agricultural biotechnology targeted to a wider, interested audience and specific working sessions focused on regulators, scientists and graduate students, with in depth discussions of risk assessment concepts and tools. These specific actions were implemented for those professionals directly involved in risk assessment activities, using a hands-on methodology. Participants included professionals from the Ministries of Agriculture and Livestock, Public Health and Social Welfare, Industry and Commerce, the National Service of Animal Quality and Health, the National Service of Plant and Seed Quality and Health, the National Institute of Forestry, the Paraguayan Institute for Agricultural Technology, the Secretariat of the Environmental and the National University of Asunción.

Seven seminars and workshops were conducted by 18 expert trainers along the entire program, both in the classroom and during visits to confined field trials. In addition to the analysis of six case studies specially developed, numerous documents and tools were provided online through the project's website. A series of e-Learning courses were also developed by the Research Foundation, available in Spanish, as a follow up tool (ILSI Research Foundation, 2017). A guide for the management of confined field trials with GM plants was an additional product of this program, responding to a request from participants.

The unifying concepts used in the risk assessment of GM crops (both environmental and food/feed safety aspects), based on Problem Formulation (Wolt et al., 2010; Garcia-Alonso, 2013), were instrumental to provide a solid scientific basis to the decision-making process.

The transition from the so called "checklist" approach to one based on problem formulation was not a trivial undertaking for CONBIO's members, as the learning curve of the regulators and the time needed to adjust are generally underestimated. The main difficulty in this aspect, was to integrate the problem formulation process within the main evaluation strategy and identifying protection goals (Garcia-Alonso and Raybould, 2014), as these should be set by federal level laws and policies, which in this case did not specify which these were.

It was also difficult for risk assessors, usually trained as researchers, to adjust to a different way of analyzing information based on regulatory science criteria and examining dossiers as a source of data that respond to risk hypotheses, rather than as an academic paper (Klimisch et al., 1997)[1].

In part as a result of this program and thanks to a deeper understanding of the scientific underpinnings of the biosafety oversight, it was possible to develop science based risk assessment instruments in Paraguay (Soerensen et al., 2014). In fact, after the completion of the program, CONBIO members issued a set of science based guidelines and application forms for experimental release in confined field trials and for the commercial authorization of GM crops, formalized through a Resolution (N° 27/2015) of the Ministry of Agriculture and Livestock of Paraguay.

Currently, the first confined field trials on private land are being carried out in Paraguay[2]. For these trials to be possible technicians had to be trained to assess the fields and monitoring systems had to be established to ensure compliance with the confinement conditions required and with adequate chain of custody processes for transgenic seeds. However, additional capacities have to be put in place as the number of trials grows.

Since its completion in 2015, the program partners have implemented periodical follow-up meetings, special sessions to discuss particular topics or to update risk assessors on new information and developments, share publications, etc.

The Partnership program also fostered open discussions among participants and with other stakeholders, contributing to enhance the level of participation of the representatives of Paraguay at regional and international meetings like OECD's working groups and other fora.

In spite of the program success in terms of capacity building, CONBIO still faces numerous difficulties. The fact that its members are not fully dedicated to risk assessment, but have other responsibilities as part of their jobs, turns the assessment into a lengthy process. Besides, members change with a certain frequency, further complicating the situation. Importantly, experts/advisors appointed to CONBIO by member institutions are experts in their fields but very seldom in risk assessment, resulting often in discussions and concerns that could be avoided with a dedicated group specialized in risk assessment. To this end, staff positions, formal trainings, hands-on experiences, inter-agency collaboration and the continued practice of biosafety assessment are key, all of which will only be possible if pertinent authorities commit to provide the needed resources.

EXPERIENCES AND LEARNINGS FROM CAPACITY BUILDING EFFORTS IN OTHER COUNTRIES

The panel on "Capacities for the Risk Assessment of GMOs: challenges to build sustainable systems," also included

[1]Regulatory science is a scientific discipline that poses risk hypotheses derived of the problem formulation step and considers policy protection goals. It generates data using standardized protocols, validated methodologies and quality assurance systems to ensure data integrity

[2]Field trials for GE crops in Paraguay have been conducted almost exclusively by IPTA (the Paraguayan Institute for Agricultural Technology). This policy was recently modified to offer developers broader options.

presentations of experiences from Argentina, Brazil, Kenya, Nigeria, South Africa, Uganda and other African countries, supported by organizations like the Food and Agricultural Organization of the United Nations (FAO), the Brazilian Agriculture Research Corporation (EMBRAPA), the Ministry of Agroindustry (Argentina), Michigan State University, ILSI Research Foundation, the International Centre for Genetic Engineering and Biotechnology (ICGEB) and the NEPAD (New Partnership for Africa's Development) African Biosafety Network of Expertise.

Martin Lema and Agustina Whelan from the Biotechnology Directorate (Ministry of Agroindustry) of Argentina, shared their experience with training programs as trainers. In particular, in the activities of the National Advisory Committee on Agricultural Biotechnology (CONABIA) as a FAO Centre of Reference for biosafety of GMOs, conducting workshops and training sessions in different countries in Sub-Saharan Africa and Ecuador, among others. The importance of training trainers and "learning by teaching others" was highlighted in this presentation. In their experience, the lack of indicators to measure efficacy needs to be addressed.

Ruth Mbabazi, Marc Heijde, and Karim M Maredia shared the capacity building efforts lead by Michigan State University for research, innovation and application of biotechnology for food security in Africa. They explained that national governments and regional economic communities (RECs) in Africa are taking positive steps in building their capacities for adoption of new technologies to enhance agricultural productivity, food and nutritional security and economic growth. This has also triggered strategic and effective public-private partnerships for translating research into practice.

Agricultural biotechnology capacity building experiences in Africa were summarized, detailing the respective roles and contributions of key continent-wide and international institutions. This presentation examined issues related to the need for technology transfer policies, practices and regulatory oversight of biotech products to enable adoption in Africa, highlighting the need to build networks to facilitate inter-country collaboration. Important challenges to be considered: the high turnover of risk assessors and difficulties to measure efficiency of capacity building initiatives.

John Teem and Libby Williams (ILSI Research Foundation) presented their e-Learning platform as a sustainable and interactive resource. While in-person workshops and meetings are an ideal way to provide education and training, several challenges can make this traditional style of capacity building increasingly difficult, including limited resources and travel constraints. By being cost-effective, interactive and accessible, e-Learning courses can be used to complement face-to-face trainings to achieve optimal learning outcomes and also be a continuing education tool.

This presentation was complemented by a capacity building case study that involved the National Biosafety Authority (NBA) of Kenya utilizing e-Learning courses developed by the Research Foundation to share biosafety information in a resource-efficient format. These resources have been translated into other languages besides English and include open access courses related to biosafety, biotechnology and food safety.

Along these lines, Dennis Ndolo, Michael Wach, Patrick Rüdelsheim and Wendy Craig introduced a curriculum-based approach to teaching biosafety through e-Learning developed by the International Centre for Genetic Engineering and Biotechnology (ICGEB). They emphasized that working in biosafety capacity enhancement incorporating approaches into activities, such that their impact becomes sustainable once funding has been depleted, can be a truly everlasting task.

Many training efforts face the limitation of one-off events: they only reach those people present at the time. However, beyond the initial effort to establish the basic content, repeating capacity enhancement events in different locations is usually not economically feasible. Also the lack of infrastructure and other resources needed to support a robust training program hinder operationalizing a "train-the-trainer" approach to biosafety training.

One way to address these challenges is through the use of e-Learning courses that can be delivered online, globally, continuously, at low cost, and on an as-needed basis to multiple audiences. Crucial to the implementation of such an e-Learning program is an approach in which the courses are intentionally developed together as a cohesive curriculum. Once developed, such a curriculum can be released as a stand-alone program for the training of governmental risk assessors or used as accredited components in graduate degree programs in biosafety, at minimal cost to the government or university. Examples from the ICGEB portfolio of biosafety e-Learning courses were presented to demonstrate these key features.

Deise Maria Fontana Capalbo from the Brazilian Agriculture Research Corporation (EMBRAPA)—Environment, shared Brazilian capacity building experiences in biosafety to support the decision-making process. The main decision body in place in Brazil is the National Biosafety Technical Commission (CTNBio), composed of 27 members and their respective alternates that hold a two-year term, renewable for up to two consecutive periods.

This presentation showed some experiences on how individuals, groups, institutions and governmental authorities acted in order to provide training and technical assistance to the decision-making bodies. There were, and still are, many types of capacity building activities in place. Different approaches incorporated a variety of forms and disciplines and many factors were taken into account (target beneficiaries, effective content according to the target audience, specific needs, integration and collaboration among the various disciplines and capacity builders). An active participation of country experts in international fora is also encouraged in Brazil.

Finally, Samuel Timpo from the NEPAD Agency African Biosafety Network of Expertise (ABNE) in collaboration with Hashini Galhena Dissanayake (Michigan State University), Joseph Guenthner (University of Idaho), Godwin Lemgo (NEPAD), and Karim Maredia (Michigan State University),

introduced institutional capacity efforts to overcome systems challenges toward building functional biosafety systems in Africa. While functional biosafety systems are critical for the safety assessment of GM crops, the development of these systems in Africa are constrained by a number of factors. Key among these factors is the lack of institutional and human capacity to design and implement biotechnology regulatory frameworks that have the capability to make science-based decision on risks and benefits of various GM crops as well as provide mechanisms for inspection, monitoring and compliance.

In view of these on-going efforts, authors attempted to identify knowledge and skill gaps through a multi-stakeholder field research carried out in six countries in Africa and discussed strategies to enhance biosafety capacity. The findings highlighted the importance of continuing capacity building programs and coordinating efforts and investments as well as broadening training modules and extending to groups beyond regulators, policy makers, and scientists. Such efforts will help minimize prevalent concerns about food and environmental risk and empower stakeholders with accurate information to counter misconceptions.

POINTS OF AGREEMENT AND RECOMMENDATIONS

- Regulatory guidelines based on sound science and risk assessment experience assist regulators in their evaluations and well-designed capacity building programs, customized to the different realities and particular needs of each country or region, can greatly contribute to build regulatory capacity.
- Biosafety systems deal with evolving concepts and technologies and need to anticipate and adjust their procedures and requirements to these advances. Therefore, a periodical revision of regulations and operations is always necessary (Vicién and Trigo, 2017). These processes require to be accompanied by permanent training cycles for risk assessors, which consider state of the art criteria and methodologies.
- For any program to be effective, it is recommended to implement early consultations and interviews with key stakeholders as well as post-training contacts for feedback and follow-up support. It is good practice to customize the programs.
- As much as these programs can give support, it will always be from the outside. Intra-agency processes must be in place in order to become self-sustainable.
- The panel concurred on the barriers to achieve sustainable systems and drew attention to the high rotation of risk assessors in some countries. The lack of dedicated professional regulators, specialized in risk assessment, is a key factor.
- To this point, the need for professional programs (specializations, degrees, masters programs) was mentioned. Academic programs are lacking in most countries and would provide a formal context to develop capacities, also offering new career development opportunities to university graduates.
- Regarding technological tools (as online courses, etc.) as much as they can help, they have to be part of integral programs, as they do not work in isolation.
- In a risk-averse society, concepts on Risk, Biosafety, Risk Science, Problem Formulation and Regulatory Sciences should be part of University curricula and even of High School programs, to educate citizens and build trust in regulatory bodies.
- Governmental commitment to support the establishment of self-sustainable systems is also essential in order to give continuity to regulatory systems, without depending on sporadic funding. To this end, it is basic to educate and engage political leaders and decision makers, on the importance of having professional, expert regulatory agencies.
- The development of metrics and indicators to assess the effectiveness of these efforts is also recommended, as this can facilitate further improvements and a better use of resources often times provided by governments, universities or international organizations.
- These metrics should be able to assess the degree of maturity of the systems, the efficiency of training efforts and their sustainability. Potential indicators can be developed around the general improvement of the decision making processes, the quality and robustness of decision documents, the implementation of consistent, transparent assessment criteria, the application of tools shared in trainings, the intra-agency processes put in place to train new regulators and keeping staffs up to date, the use of inter-agency consultations /collaborations, etc.
- Panel members also agreed on the importance of personal experience, as well as teaching others, as great learning experiences. The common message was around the value of partnerships.
- In summary, it takes time to design regulatory frameworks and to develop expertise to put them into practice and conduct periodical updates. This is an everlasting process and as technologies and frameworks evolve, regulators need to be prepared to keep up with these changes in a dynamic and continuous cycle.
- As valuable as the efforts made by diverse organizations might be, these will fail to build sustainable systems without country policy decisions to support the development of dedicated, professional and transparent regulatory bodies that can focus on the high responsibility of protecting environmental and human health.

This has been a first attempt to share experiences and identify barriers to sustainable systems. It would be desirable to follow up on these discussions in order to put some of these recommendations (like formal training opportunities or the development of metrics), into practice.

AUTHOR CONTRIBUTIONS

All authors participated in the drafting of this paper as individual experts in their fields, and the authors are solely responsible for

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Policy-Led Comparative Environmental Risk Assessment of Genetically Modified Crops: Testing for Increased Risk Rather Than Profiling Phenotypes Leads to Predictable and Transparent Decision-Making

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We describe two contrasting methods of comparative environmental risk assessment for genetically modified (GM) crops. Both are science-based, in the sense that they use science to help make decisions, but they differ in the relationship between science and policy. Policy-led comparative risk assessment begins by defining what would be regarded as unacceptable changes when the use a particular GM crop replaces an accepted use of another crop. Hypotheses that these changes will not occur are tested using existing or new data, and corroboration or falsification of the hypotheses is used to inform decision-making. Science-led comparative risk assessment, on the other hand, tends to test null hypotheses of no difference between a GM crop and a comparator. The variables that are compared may have little or no relevance to any previously stated policy objective and hence decision-making tends to be *ad hoc* in response to possibly spurious statistical significance. We argue that policy-led comparative risk assessment is the far more effective method. With this in mind, we caution that phenotypic profiling of GM crops, particularly with omics methods, is potentially detrimental to risk assessment.

Keywords: risk assessment, genetically modified crops, regulatory policy, problem formulation, profiling, hypothesis testing

INTRODUCTION

Regulatory risk-management of GM crops often uses comparative risk assessment to inform decision-making. Decisions may include whether to allow cultivation or importation of a particular crop in the relevant jurisdiction, and whether any conditions need to be placed on those uses if they are permitted. Comparative risk assessment contextualizes the risk by comparing the risks posed by the cultivation of the GM crop with the risks posed by the cultivation of the non-GM counterpart. If the risk assessment indicates that cultivating a GM crop poses no greater environmental risk than cultivating the non-GM counterpart, then it might be thought that cultivating the GM crop

poses no unacceptable risk. However, judging the acceptability of a risk goes beyond the scientific comparison of relative risks. In order to make this point, we discuss definitions of risk, opportunity and acceptability. We concentrate on environmental risk assessment and GM crops, but our discussion is pertinent to risk assessment and decision-making more generally.

Defining Risk and Opportunity

Risk may be expressed as a combination of the likelihood and severity of harm that may arise from hazardous properties of a proposed activity. Environmental risk assessors often think of risk in terms of the potential exposure to the hazard that can cause a harm, where potential exposure is the expression of likelihood. Seriousness of harm is related to the degree of hazard, but also contains subjective elements (see below). Risk is usually difficult to quantify precisely, and most risk assessments rely on qualitative assessments and expert judgment. If severe harm is likely, risk is high; and if the most serious conceivable effect is trivial and unlikely, then risk may be regarded as negligible. However, even a tiny probability of a harmful effect may be regarded as high risk if the harmful effect is serious. A severe decline in the population size of an endangered or iconic species might be one such effect. Risk may also be regarded as non-negligible if low severity events are predicted to occur frequently (e.g., Slovic, 1999).

Similar considerations apply to the opportunities that may arise from an activity. Opportunity is high if very valuable benefits are likely to arise, such as shifts to more sustainable agricultural practices as have been seen in Canada with the widespread adoption of GM herbicide-tolerant (GMHT) canola varieties. Use of tillage by growers prior to seeding for weed control for canola appears to have been eliminated and the significant shift to minimum and zero tillage systems has reduced soil erosion, resulted in higher carbon sequestration in production areas, reduced the need for herbicide applications and created net economic benefits for growers (Gusta et al., 2011; Smythe et al., 2011). Opportunity is negligible if the most valuable benefit is unlikely and of low value, such as cultivation of a GM drought tolerant crop in an area where precipitation is almost never yield limiting. Opportunity may still be regarded as high if beneficial effects are unlikely, but would be hugely valuable if they arose. The reduction of a non-target effect to a highly beneficial or iconic insect species that may only rarely co-occur with crop production could be considered as highly beneficial. This may occur if cultivation of the GM crop reduces the spraying of pesticides, either directly through endogenous insect protection or indirectly by carrying a disease tolerance that reduces the need to spray for an insect vector of the disease. Significant opportunity may also accrue from frequent events of relatively low value.

Judging the Acceptability of Risk

Judging the acceptability of risk requires a method to weigh the opportunities against the risks of the activity under consideration (Sanvido et al., 2012). Under ethical decision-making, if a risk exceeds an acceptability threshold, then the risk is unacceptable regardless of the size of the opportunity. Under utilitarian decision-making, the course of action posing the highest net opportunity—the opportunity minus the risk—must be selected. It follows that even severe risks may be acceptable provided the opportunities are high enough, and that an increase in risk many be acceptable provided it is outweighed by increased opportunity.

In practice, determining the acceptability of risk for the cultivation of a GM crop is made difficult by the need to balance complicated sector needs with a broader public good. The 1993 Canadian Regulatory Framework for Biotechnology (Industry Canada, 1998; Gabler, 2008), for example, attempts to articulate guiding principles for how decisions could be structured. The framework captures the idea that any regulatory decisions should enable innovation, but also protect the environment and the health and well-being of citizens. Governments often have competing internal interests where departments of environment may view the opportunities for cultivating GM crops differently from Departments of Agriculture who see the acceptable risks and benefits of agriculture with a more commercial perspective.

Determining whether an activity poses acceptable risk requires several difficult judgments. First, one must decide what would be regarded as harmful effects of the activity and what would be regarded as beneficial effects. In addition, one must decide how to judge the severity of harm and the value of benefits. While science may be used to limit the scope of discussions of harm and benefit to plausible effects of the proposed activity (Raybould, 2010a), the designation of an effect as harmful, beneficial or neither, and the severity and value ascribed respectively to harmful and beneficial effects of a particular size relies on non-scientific criteria. These criteria may be based on personal values, an organization's objectives or public policy depending on who will make the decision. For brevity, hereafter we refer to these non-scientific criteria as "policy objectives."

The second difficult judgment is how one will weigh risk and opportunity. One must consider whether certain effects should be unacceptable regardless of the size of the opportunity or whether the largest net opportunity will always be the preferred option. In addition, one will need a method for evaluating net opportunity when benefits and harms may be very different; how, for example, does one evaluate the net opportunity if growing a certain crop is expected to increase yield but reduce other ecosystem services (de Groot et al., 2010).

The above considerations show the importance of setting clear policy objectives in order to ensure that the scientific parts of risk assessment answer questions that are useful for decision-makers rather than questions that scientists may find interesting (Hill and Sendashonga, 2003; Evans et al., 2006). In practice, even with policy direction, such as a policy objective on the conservation of biodiversity, risk assessors rely on professional judgment when they weigh evidence in what is often a qualitative process and make a number of "micro policy judgments" while conducting the assessment. Indeed, the promotion of "science-based risk assessment" (= science-led in our terms) (e.g., Andow and Hilbeck, 2004; Kuntz et al., 2013) could lead to the mistaken and pernicious idea that it is desirable to eliminate consideration of policy objectives and judgment from risk assessment. Such thinking is almost guaranteed to produce controversy and paralyze decision-making (e.g.,

Raybould, 2010b). Instead, "policy-led risk assessment" ought to be the aim (**Figure 1**).

In this article, we explore the implications of a change of emphasis from science to policy on two aspects of comparative environmental risk assessment of GM crops that are of current interest: problem formulation and the use of profiling data from various omics techniques. While we focus on regulatory decision-making about GM crops, our remarks are relevant to all crops with novel phenotypes, however they are produced, and to other types of decision-making, such as choosing which products to develop (Macdonald, 2014).

PROBLEM FORMULATION

Risk Hypotheses and Decision-Making Criteria

In essence, regulatory risk assessments should test hypotheses that help risk managers to make good decisions about whether to permit particular activities. Problem formulation is the process by which these risk hypotheses, and plans to test them, are devised. While we concentrate on environmental risk posed by the cultivation of GM crops, our comments are relevant to any regulatory decision-making that makes use of risk assessment.

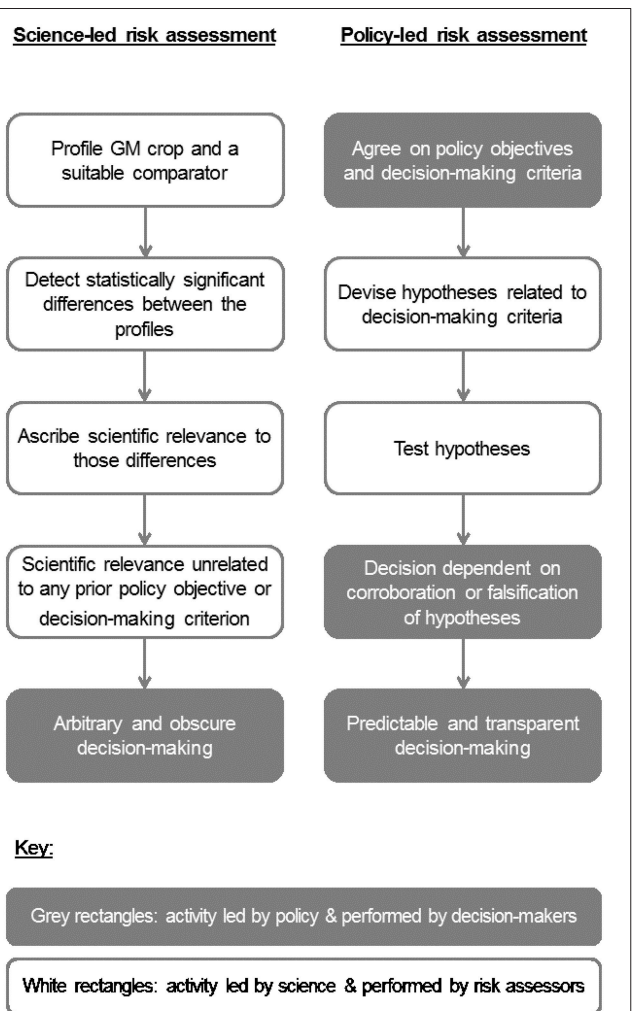

FIGURE 1 | Conceptual models of science-led and policy-led risk assessment.

In regulatory environmental risk assessment, decision-making criteria should relate to the probability and severity of environmentally harmful effects arising from the proposed activity covered by the regulations. In the case of GM crops, the proposed activity will be cultivation of a specified GM crop in a particular place, perhaps with other stipulations such as whether certain crop-protection chemicals will be applied to the crop. The definition of what is harmful is a matter for the risk managers based on their interpretation of the policy objectives of the legislation that the regulations are designed to implement.

At their most conservative, the risk hypotheses will be that no harmful effect will result from the proposed activity. If these hypotheses are corroborated under rigorous testing using information from reputable sources, including data from laboratory or field tests, the risk managers can be confident that the proposed activity poses negligible risk, and then use that conclusion in their decision-making. Less conservative risk hypotheses acknowledge the probability and contextualize the impact of any harmful effect; that is, the hypotheses under test would be that the risk does not exceed a threshold of acceptability. The threshold may be set to be the same as the risk posed by similar activities, or higher risk could be tolerated if the activity provides greater opportunities; for example, greater risk might be acceptable for cultivation of a GM crop that provides higher yield or improved quality than the crops it will replace. Rigorous corroboration of the hypotheses would indicate that the risks could be placed in the context of those from comparable activities, such as the cultivation of a non–GM crop that has a similar trait, even though the risks may not be negligible. That conclusion would contribute to decision-making.

Placing Risks in Context of Current Practice

In theory, regulations could specify that certain effects are harmful if they are caused by the cultivation of GM crops but are not harmful if caused by other activities. However, such definitions of harm would violate accepted standards of good regulatory practice. The OECD (2014) describes eight Principles of Regulation, and defining effects as harmful only if they are caused by GM crops would violate at least three of them: Principle 2 that regulations must have a sound legal and empirical basis; Principle 4 that regulations must minimize market distortions; and Principle 7 that regulations should be consistent with other regulations and policies. Hence, definitions of acceptable risk for GM crops should consider what is regarded as acceptable for other agricultural practices.

Many publications have concluded that conceivable harmful environmental effects from cultivating GM crops are of the same type as those from growing non-GM crops (e.g., Tiedje et al., 1989; NRC, 2002; Perry et al., 2004; Lemaux, 2009). Hence, 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 the current practice that cultivation of the GM crop may replace. Similarly, a hypothesis that growing a certain GM crop will poses no unacceptable risk, is really a hypothesis that any increase in risk caused by growing the GM crop will be acceptable, either because the increase falls below a threshold of acceptability or because the additional opportunities created by growing the crop are worth the risk. As "no additional harm" sets a higher standard than "no unacceptable increase in risk," testing a hypothesis of no additional harm may be regarded as rigorous testing of a hypothesis of no unacceptable increase in risk provided other factors that determine acceptability of risk, such as the size of the opportunity, are unchanged.

A hypothesis that growing a GM crop will cause no unacceptable increase in risk is useful in a least three respects. First, corroboration or falsification of this hypothesis is valuable to risk managers. Second, it shows that GM regulation follows the Principles of Regulation by not treating GM crops differently from other agricultural practices. Finally, it is useful to risk assessors, because if "unacceptable risk" is sufficiently operationalized, risk assessors have clarity about the data they need in order to conduct the risk assessment, namely data that test the hypothesis of no unacceptable risk.

Consider a proposal to cultivate a new variety of GMHT canola that is likely to replace long-standing cultivation of a non-GM ("conventional") canola. Also, suppose that the effects of recommended herbicide applications to the GMHT canola fall under regulations covering GM crops and the effects of recommended herbicide application to the conventional canola are covered by pesticide regulations. A possible effect of switching from conventional canola to the GMHT canola is a change in the abundance and species diversity of weeds owing to variation in their sensitivity to the different herbicides used on these crops (e.g., Perry et al., 2004; Wilson et al., 2007). In assessing the risks posed by cultivating the GMHT canola, the Principles of Regulation suggest that it would be unreasonable to compare the weed flora in the GMHT canola regime with the weed flora if no herbicides were used; the comparison ought to be with the conventional herbicide management.

Assessing Risks Rather Than Measuring Differences

Identifying a fair comparator is only a partial solution to the problem of formulating a useful risk hypothesis. Countless changes in the weed flora are theoretically possible when switching from conventional to GMHT weed management. Science-led risk assessment (**Figure 1**) might approach this problem by setting up multiple field trials at many sites over many years to measure the change in the weed flora when GMHT replaces conventional management; in effect, the hypothesis under test would be one of no difference between the weed floras of conventional and GMHT canola.

Comparing weed diversity and abundance between conventional and GMHT canola will almost inevitably reveal numerous statistically significant differences (e.g., Heard et al., 2003a,b), with the number limited only by the size of the experiments, the sensitivity of the measuring techniques and the imaginations of the researchers in devising ways to categorize difference. However, few or even none of these differences may have any relevance to regulatory policy objectives. Consequently, cataloging differences is at best an inefficient way to conduct risk assessment, because effort is wasted on measurements of no value for decision-making. At worst it is ineffective and potentially counterproductive because decisions are made *ad hoc* in response to statistical significance, which can easily be spurious when many variables are measured (Benjamini and Hochberg, 1995; Leek et al., 2017), rather than after serious consideration of what the objectives of agricultural and environmental policies ought to be. We could call this behavior PARKing—*P*olicymaking *A*fter the *R*esults are *K*nown—based on Kerr's (1998) term HARKing for *H*ypothesizing *A*fter the *R*esults are *K*nown.

Policy-led risk assessment would approach the problem by defining, at the very least, general trends that would be regarded as harmful changes in the weed flora; harmful meaning detrimental to achieving policy objectives. One might define harm of cultivating the GMHT canola as an increase in the abundance of specific species of economically damaging weeds, or a decrease in abundance of specific species that may have aesthetic or nature-conservation value, compared with their abundance under conventional management (e.g., Pimentel et al., 2001). Another option would be the incorporation of some decision-making criteria into the definitions; thus, one might define the threshold of unacceptable harm as a 50% increase in the abundance of noxious weed X or as a 25% decrease in the abundance of endangered species Y.

Prior definition of decision-making criteria means that experiments can be designed to rigorously test risk hypotheses. One could envisage, for example, testing a hypothesis that the abundance of noxious weed X will not increase by more than 50% by testing a hypothesis that it is at least as sensitive to the herbicide that will be applied to the GMHT canola as it is to the herbicides applied to conventional canola. Such a targeted test of a policy-relevant hypothesis would be entail vastly more efficient and effective parameters for data collection than would untargeted comparisons of the weed floras of GMHT and conventional canola.

With best practices, risk assessors will contextualize the risks for cultivating the GMHT canola and compare that with the harm from the cultivation of conventional canola. In the risk assessment, the risk assessor will consider that cultivation of a monoculture and the management of a crop in an agricultural production system reduces biodiversity and has an impact on the environment. The crop plant itself has a suite of traits that result in the production of compounds that create environmental effects and influence ecosystem services. In the comparative risk assessment, the risk assessor will evaluate the relative impacts of the two phenotypes and evaluate whether the addition of the new trait creates harms that exceed those already imposed by the cultivation of the existing crop. In this scenario, the evaluation does not insist the results of growing the two crops be identical, only that the probability or severity of a harm is not increased.

Policy-led risk assessment can target risk management to make interventions in order to realize benefits and reduce harms. In testing the risk hypothesis that the endangered species Y will

not decrease by more than 25%, testing may reveal that the species is more sensitive to the GMHT herbicide than to the conventional canola herbicide. This finding could trigger a search for changes to management techniques that ensure weeds are still adequately controlled while minimizing exposure of species Y to the herbicide, perhaps by altering the proposed timing, rate or method of its application (e.g., Thompson et al., 1991). In contrast, unfocussed risk assessment may reveal potential changes in the abundance of numerous species without any attempt to contextualize the risk. Faced with such a finding, risk managers may simply refuse to approve the GMHT canola (Sanvido et al., 2011), thereby foregoing opportunities and not necessarily reducing risk—although they may have reduced the probability of change.

In summary, problem formulation for comparative risk assessment of GM crops should consider two important elements. First, the comparison should be consistent with the Principles of Regulation. The effects of using the GM crop should be compared with agricultural practices that these uses will replace. Second, the selection of the hypotheses to be tested in the risk assessment should always be policy-led and informed by science. Policy-led risk assessment will guide risk assessors to develop hypotheses of known relevance to the final regulatory decision and suggest experiments that are required to improve decision-making rather than satisfying scientific curiosity. The combination of hypotheses based on prior agreement of decision-making criteria and rigorous testing maximizes the chances that risk managers will make decisions that fulfill agricultural and environmental policy objectives. Risk communication will also be improved. Science-led risk assessment, on the other hand, leads to PARKing: *ad hoc* decision-making based on whatever differences happen to reach statistical significance in comparisons of many variables. These decisions are unlikely to meet wider policy objectives. They are also likely to create controversy because decisions appear to be fixed by selecting particular data rather than after a debate about what the objectives of policy ought to be (e.g., Sarewitz, 2004).

PROFILING IN RISK ASSESSMENT

In the example above, we proposed that rigorous testing of targeted hypotheses is a more efficient and effective approach to risk assessment than are untargeted tests of null hypotheses of no difference between a GM and a non-GM cropping system. The latter approach makes use of profiling—the characterization of a system by describing a combination of many of its attributes.

Historic and Current Use of Profiling in Risk Assessment

Profiling of GM crops is used widely in risk assessment. Compositional analysis typically tests for statistically significant differences between the GM crop and a near-isogenic comparator variety in the amounts of 60–80 nutrients and anti-nutrients (Herman and Price, 2013). Phenotypic characterization compares 30 or more aspects of germination, plant growth and development, morphology, reproduction, disease and pest damage, and attributes of grain or fiber quality depending on the crop (Horak et al., 2007). The aim of these studies is to identify differences between the GM crop and its comparator that need further evaluation in order to characterize risk to human and animal health and to the environment from using the GM crop (Kuiper et al., 2001; Nap et al., 2003).

Although not routinely required for regulatory testing, profiling of GM crops can also be carried out at the molecular level, using transcriptomics, proteomics or metabolomics (Kuiper et al., 2003). The value of these methods, along with characterization of the epigenome, for crop improvement has recently been discussed by the National Academies of Sciences, Engineering and Medicine (NAS, 2016). Our purpose here is not to evaluate the technical feasibility of molecular profiling, but to discuss whether profiling approaches generally are valuable in risk assessment of GM crops.

A claimed advantage of profiling methods is that they are unbiased (Kuiper et al., 2003). They make no assumptions about how the GM crop might differ from its non-GM counterpart. In addition, unbiased approaches make no judgment about what differences might be important in indicating that using the GM crop may pose greater risk than similar uses of the comparator. Hence, profiling approaches are science-led evaluations of potential differences with all the problems that entails (**Figure 1**).

In the early days of GM crop development, there was significant uncertainty about the extent to which transformation of plants could lead to unintended changes. Hence, compositional and phenotypic profiling of GM crops made sense as methods to explore the extent of these changes: testing the hypothesis that transformation introduces no unintended changes was a useful tool for basic research into the effects of transgenesis and also for risk assessors struggling to characterize products of new technology.

In retrospect, however, there was always a need to ensure that these studies were placed in context when used to inform the risk assessment. In practice, this has generally been the case when a GM crop and its non-modified counterpart are compared. For example, as changes in the nutritional value of a crop could be harmful to human and animal health, the risk assessor determines whether the amounts of key nutritional components are statistically different between the GM and non-GM comparator. If statistically significant differences are identified, the assessor will ask whether the amounts in the GM crop fall into the normal range for that crop. If they do, the differences will generally be disregarded.

It is important to recognize that comparing nutrients is policy-led risk assessment because protecting human and animal health is a policy objective. To keep the risk assessment policy-led, however, it is important that the substances tested really are determinates of health. If the most extreme conceivable change in the amount of a substance would have no material effect on health, then that substance should be of no concern for policy-led risk assessment, and comparing its concentration in the GM and non-GM crop should not be necessary to determine risk.

Without prior definitions of important changes, science-led profiling can encourage the idea that producing more data

inevitably leads to better risk assessment. Statistically non-significant comparisons of thousands of substances may appear to be a more convincing demonstration of negligible risk than is the lack of difference in a few key nutrients. However, unless it is possible to specify values of particular variables that would show a policy-led risk hypothesis to be false, the data are of no relevance for drawing conclusions about risk. Finally, profiling may also understate the importance of policy in risk assessment and decision-making. It seems to promote the idea that if sufficient data are collected, uncertainty will be diminished and the "correct" policy toward the use of GMOs will become obvious.

Profiling Using Omics Methods

The introduction of molecular profiling methods into regulatory risk assessments would only increase the pervasiveness of unfocussed data generation rather than policy-led attitudes to risk assessment. Additional data generation will often pose questions for which there are no ready answers leading to a continuing need to produce yet more data. The ability to find differences between a GM crop and its non-GM comparator is virtually limitless, creating endless opportunities for PARKing. Advocates of molecular profiling may argue that the methods could show that variation between GM and non-GM plants as a class is insignificant compared with variation among non-GM plants. However, this misses the point. The purpose of regulatory risk assessment is not to make general points about a technology or class of products, it is to evaluate whether the risks posed by a specific use of a specific product are acceptable. Acceptability of risk is ultimately a policy decision, and anything that promotes policymaking as an *ad hoc* response to possibly spurious statistically significant differences, rather than careful deliberation about delivering agreed societal objectives, should be discouraged.

Finally, our point is not that omics methods can never have value in regulatory risk assessment. If measurements of specific transcripts, proteins or metabolites are a good test of a hypothesis that a given use of a given GM crop does not pose an unacceptable increase in risk, then the measurements may have value for regulatory decision-making. However, using the methods simply to create profiles will be a serious impediment to moving from science-led to policy-led risk assessment and decision-making.

CONCLUSIONS

Comparative risk assessment is a valuable method for making risk assessment tractable, provided that it is policy-led rather than science-led. Ideally, policy-led comparative risk assessment for a GM crop would define effects that comprise unacceptable increases in risk from its use. The comparison would be with the acceptable effects of a similar crop in a similar agricultural system that is likely to be replaced by use of the GM crop.

Defining an unacceptable increase in risk enables the formulation of testable hypotheses for risk assessment. At their most conservative, the hypotheses will be that certain effects are no more likely to occur, and if they do occur, are no more severe than those caused by use of the crop that will be replaced. Only data that test such hypotheses, that is, are able to show them to be false, are useful for such policy-led risk assessment.

The alternative method of comparative risk assessment dispenses with policy objectives and makes numerous tests of the null hypothesis that the GM crop does not differ from the crop that it will replace. Such "science-led" risk assessment makes no judgment about the importance of the variables being measured. Proponents of this method of risk assessment see this unbiased nature of the risk assessment as a strength (e.g., Kuiper et al., 2003).

However, while lack of bias in testing a hypothesis is a virtue in risk assessment, as in all basic and applied science, lack of bias in selecting the hypotheses to be tested is a grave weakness: we should be strongly biased toward hypotheses that help decision-making and realization of policy objectives. Without this bias, policy may be formulated in response to trivial differences, perhaps influenced by ill-informed indignation that a GM crop, unsurprisingly, differs from a non-GM comparator in some respect. It is this very lack of bias that we believe makes science-led risk assessment vastly less effective than the policy-led alternative.

In advocating policy-led risk assessment, we do not underestimate the difficulties agreeing on policy objectives. Disagreement about what comprise beneficial or harmful effects of using certain GM crops is rife, even within organizations that develop and regulate them. However, sooner or later policy objectives have to be set in order to make decision-making feasible and hence risk assessment efficient and effective. While defining these objectives may be controversial, such controversy is likely to be less than that produced by making policy *ad hoc* in response to possibly spurious statistically significant differences identified by untargeted profiling methods. Ultimately, decision-makers have to decide based on their individual or organizational policy objectives. This responsibility cannot be outsourced to statistical algorithms processing vast amounts of profiling data.

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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A Curriculum-Based Approach to Teaching Biosafety Through eLearning

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Anyone working in biosafety capacity enhancement faces the challenge of ensuring that the impact of a capacity enhancing activity continues and becomes sustainable beyond the depletion of funding. Many training efforts face the limitation of one-off events: they only reach those people present at the time. It becomes incumbent upon the trainees to pass on the training to colleagues as best they can, whilst the demand for the training never appears to diminish. However, beyond the initial effort to establish the basic content, repeating capacity enhancement events in different locations is usually not economically feasible. Also, the lack of infrastructure and other resources needed to support a robust training programme hinder operationalizing a "train-the-trainer" approach to biosafety training. One way to address these challenges is through the use of eLearning modules that can be delivered online, globally, continuously, at low cost, and on an as-needed basis to multiple audiences. Once the modules are developed and peer-reviewed, they can be maintained on a remote server and made available to various audiences through a password-protected portal that delivers the programme content, administers preliminary and final exams, and provides the administrative infrastructure to register users and track their progress through the modules. Crucial to the implementation of such an eLearning programme is an approach in which the modules are intentionally developed together as a cohesive curriculum. Once developed, such a curriculum can be released as a stand-alone programme for the training of governmental risk assessors and regulators or used as accredited components in post-graduate degree programmes in biosafety, at minimal cost to the government or university. Examples from the portfolio of eLearning modules developed by the International Centre for Genetic Engineering and Biotechnology (ICGEB) are provided to demonstrate these key features.

Keywords: biosafety, eLearning, risk analysis, distance education, curriculum, biorisk management, food safety, environmental safety

INTRODUCTION

Modern biotechnology refers to a number of techniques that involve the intentional manipulation of genes in a predictable and controlled manner, but beyond normal breeding barriers, to generate changes in the genetic make-up of an organism. Such techniques offer great potential for meeting critical needs for food, agriculture, health, and sustainable socio-economic development. However, since modern biotechnology may result in the production of novel organisms, many regulatory authorities worldwide regulate these products as potential biohazards, in an effort to ensure human and environmental safety. Consequently, risk assessments are required before any activity involving them is performed, and only when safety has been duly demonstrated, can they be made commercially available. There are also a number of other organisms that can be exploited by humans for a range of activities; some of which, if not properly handled, could cause harm, either directly, or indirectly, resulting in considerable health, environmental, social, and economic losses. In addition, there are increased threats from the spread of weeds, pests, and pathogens, due to the rapid surge in global movement of people, goods, and organisms coupled with the growing security interest in the potential of organisms as agents for bioterrorism. In light of these concerns, national legislation, as well as various international agreements such as the International Health Regulations, 2005; United Nations Security Council Resolution 1540; the Biological and Toxin Weapons Convention, 1975 (Sture et al., 2013); the International Food Standards of the Codex Alimentarius Commission 1999 (FAO, 1999); and the Cartagena Protocol on Biosafety to the Convention on Biological Diversity, 2000 (CBD, 2000), require that measures—including the provision of relevant education and training—are put in place to prevent harm from biological material. As a result, there is global recognition for the need to develop international biosafety and biosecurity capacity, spanning many sectors, and disciplines, and especially in the developing world and for those countries with economies in transition. Biosafety and biosecurity are related concepts, in that both focus on measures to ensure protection from adverse effects associated with biological material. While biosafety pertains to the protection of human health and the environment from the possible adverse effects of the products of modern biotechnology (CBD, 2000) and is generally used to describe frameworks encompassing the policy, regulation, and management to control potential risks associated with the use of the technology (FAO, 2006), the term biosecurity is most commonly used to refer to mechanisms to establish and maintain the security and oversight of pathogenic microorganisms and toxins to prevent possible misuse; with due attention to all relevant information, knowledge, processes, practices, and equipment associated with potentially or actually hazardous biological material (Sture et al., 2013). An integrated biosafety and biosecurity training curriculum would therefore enable countries to meet their obligations under the above international agreements, and at the same time build their national capabilities to effectively address their own biorisk threats.

Over the years, the Biosafety Group of the International Centre for Genetic Engineering and Biotechnology (ICGEB) has been addressing this need through the development and implementation of a comprehensive educational and training programme in biosafety. This programme has led to the development of highly-skilled and trained personnel whom regulatory authorities can rely upon to ensure there is full and balanced consideration of biosafety issues in pursuing appropriate uses of modern biotechnology. The training programme has involved *inter alia*, the development and establishment of post-graduate programmes at both the Universities of Aberystwyth (UK) and Adelaide (Australia), crucially with essential biosafety components, together with the provision of financial support to a number of African regulatory officials to undertake the programmes. In addition, the Biosafety Group has organized numerous workshops around the world providing basic and advanced training and mentorship to further develop biosafety regulatory capacity.

However, the impact of these face-to-face approaches may be difficult to sustain once funding has been depleted, and repeating such training events in different locations is both difficult and costly. As a result, the Biosafety Group explored alternative learning environments that would provide flexibility and remain available for a prolonged period. One option that was explored was to provide training sessions as webinars, e.g., the American Biological Safety Association regularly organizes webinars (https://absa.org/online-education/) on selected biosafety topics. The advantages offered by this approach is that, compared with face-to-face trainings, a webinar provides broader accessibility, since students and trainers are not required to travel. Also, costs associated with logistics are substantially reduced once the webinar platform has been set up. Nevertheless, this approach still limits the interaction to the single occasion when students and trainers meet virtually.

Another distance learning approach that was investigated involves the use of online discussion fora. For example, the international eLearning postgraduate course "Biosafety in Plant Biotechnology" offered by IPBO and Ghent University (https://studiekiezer.ugent.be/postgraduate-studies-in-biosafety-in-plant-biotechnology-en) combines on-campus training with complementary distance learning in the form of assignments and participation in online discussion groups/chat sessions between students and trainer. This latter aspect allows individual follow-up and in-depth elaboration of specific topics however, although it has less time constraints and is less dependent on punctual access to the Internet, it does limit the active involvement of both the trainers and students to a fixed period.

Other online courses include the Biosafety Practitioner Course (http://www.bti.ed.ac.uk/courses/) and the Professional Course in Biorisk Management (http://www.bti.ed.ac.uk/biorisk-management/), both provided by the Biosafety Training Institute of the University of Edinburgh, UK. These courses are however only offered during specific time periods which limits access. The Centre for Biosecurity of the Public Health Agency of Canada and the Office of Biohazard Containment and Safety of the Canadian Food Inspection Agency offers an eLearning course on

Principles of Laboratory Biosafety (https://training-formation.phac-aspc.gc.ca/course/index.php?categoryid=2) which focusses on biosafety in laboratory and containment facilities.

While considering these options, the Biosafety Group began to expand the reach and sustainability of its training programmes, by developing an online eLearning platform for biosafety training. The term eLearning is used here to describe a broad spectrum of internet-based education. By choosing to locate the ICGEB eLearning platform on the cloud (https://showcase-icgeb.elearning.it), it allows users to access content from anywhere with a network connection. It also means that the updating of local IT hardware and software no longer presents technological and financial hurdles, whilst also liberating local providers from bandwidth limitations—a frequent constraint in developing countries. Such a platform therefore ensures that ICGEB biosafety training can be delivered online, globally, at low cost, and on an as-needed basis to multiple audiences. The modules are maintained on a remote server and made available through a password-protected portal that delivers the module content, administers exams, and provides the administrative infrastructure to register users and track their progress. The modules in the ICGEB eLearning platform are being used as stand-alone courses for the training of risk assessors, and as components in post-graduate degree programmes in biosafety, at minimal cost to the hosting government or university.

DEVELOPING A COMPREHENSIVE AND COHESIVE BIOSAFETY CURRICULUM

In order to ensure that all of the key biosafety concepts and training elements are covered, it is necessary that such a training programme is developed as part of a broad-based, cohesive, and comprehensive core curriculum, encompassing all elements common to biosafety and biosecurity regulation. In the development of the eLearning platform, ICGEB has therefore worked closely with established biosafety regulatory offices, institutions, and individuals with strong credentials in biosafety research, education and training, policy, and regulation. Regulatory offices have been involved in the development of the programme to ensure that it has relevance to the needs of GMO regulatory bodies. This element gained increasing importance in preliminary meetings with potential beneficiaries, principally African regulators, who reported that previous efforts to develop such a programme came to naught as they were not tailored to end-user needs. Therefore, from these initial consultations, the following topics were prioritized for development into core eLearning modules:

- **Biosecurity and biosafety**—With the focus on identified biological hazards requiring obvious containment, this introductory module was developed to provide context-setting information, in order to understand the nature of hazards and uncertainties associated with biological material (comprising both GMOs and non-GMOs [principally]). Biosafety and biosecurity are presented as complimentary components of biorisk management, with an especial focus on established practices specific to biosecurity issues.
- **Biosafety regulatory frameworks**—This module introduces users to the different components of a regulatory framework aimed at safeguarding human health and the environment when working with biological material and/or biotechnology applications. A functional framework comprises: legal documents (such as policy, legislation, guidelines, and decisions), authorities, advisory bodies, and enforcement mechanisms. The module guides the user through such a framework, and highlights the importance that it should reflect government policy, which should be coherent with societal priorities and values.
- **Risk analysis**—The purpose of the risk analysis module is to impart knowledge and skills to users to conduct evidence-based risk analysis. At the end of the module, users are able to assess risk using science-based approaches, develop appropriate management options for identified risks, and effectively communicate the risk and appropriate management options to the public and relevant authorities.
- **Containment and confinement of organisms**—Containment and confinement measures can be used when a risk assessment identifies risks that must be managed (e.g., when handling pathogens) or when there is uncertainty on safety (e.g., when developing certain products of biotechnology). This module guides users through the selection, implementation, and verification of various containment and confinement approaches.
- **Environmental safety**—New biological elements can have various impacts in ecosystems. It is necessary that such potential impacts are determined and addressed prior to introducing derived products into the ecosystem. This module focuses on the identification of the most common sources of potential environmental harm from novel biological organisms, as well as the types of analyses that need to be conducted in order to assess the potential risks of such organisms.
- **Food safety**—The objective of this module is to enable users to understand how the safety assessment of food is undertaken in relation to various food-related risks. Internationally-agreed procedures for food safety assessment, as published by Codex Alimentarius, are described in this module and their application discussed. Scientific and public policy issues of relevance to food safety including labeling, traceability and identity preservation of food commodities are also covered.
- **Socio-economic considerations in biosafety decision-making**—Although not a mandatory requirement in decision-making processes in many countries, there is increasing international discussion on the need to consider a broader range of issues, beyond those concerning the environment and health, when assessing the use of novel technologies. This module addresses the possible socio-economic implications of technology adoption, including how these should be addressed within a decision-making context.

As we hope is apparent from the preceding module descriptions, each has been developed to cover key elements and approaches not only specific to biosafety and the regulation of GMOs, but extended to also cover complementary regulatory areas

of technology and science (especially, biological). In this way, the portfolio of modules is supportive of the harmonization of similar regulatory approaches, when applicable, as well as being of broader utility to post-graduates seeking employment across government regulatory agencies. The curriculum will be continuously revised as new issues and information emerge so that it stays relevant to emerging biosafety and biosecurity concerns and approaches.

DESIGN ELEMENTS OF THE ICGEB ELEARNING PLATFORM

In the design of the eLearning platform, ICGEB needed to address the fact that even high-quality educational content can be undermined by a poorly designed platform. In addition, with today's tech- and media-savvy users, it is not sufficient to simply upload recordings of lecturers giving PowerPoint presentations (e.g., podcasts) onto a website in order to create a meaningful eLearning experience. Considerable thought and effort was invested in the design of the platform itself, to ensure that it not only enhanced the content, but also provided a variety of learning and testing modalities, and was also easy and cost-efficient to administer.

Design Elements to Enhance the Learning Experience

Several features were customized for the eLearning platform to provide a sophisticated and robust interactive learning experience. These focused on:

- *Use of multimedia:* Using a variety of media (multimedia) in a learning experience stimulates and encourages users to think more reflectively and helps in maintaining their interest in the learning material (Permanasari et al., 2016). As with many complex scientific topics, the concepts of biosafety are therefore best conveyed to users by exploiting the array of multimedia tools available, and as such, elements were therefore selected within the eLearning platform to accommodate a wide range of media formats. Users have a range of learning styles, and merely displaying text on the screen is almost never a good approach to teaching or to fostering user engagement (Truong, 2016), so the eLearning platform was designed to incorporate static and animated graphic elements, video lectures, interactive diagrams, and external reading material and other resources. The module developer is then free to incorporate these various elements, as needed, to best present the material to the users. For example, a video lecture describing the key features of a Biosafety Level 3 laboratory can be complemented with an interactive diagram of such a lab, in order to reinforce the key points from the presentation and help the users to understand that the features of a BL-3 lab are specifically designed to work together as a system in order to ensure containment.
- *Compatibility with mobile technology:* The specific eLearning platform was selected in the acknowledgement that the primary access by users may very well be through the use of mobile technology, e.g., tablets or even smartphones (Viberg and Grönlund, 2017). The platform itself, as well as the presentation of the content, had to support access by a variety of devices, so that each user would always have a high-quality experience, regardless of the device used. This required careful consideration of issues such as font sizes, resolution of graphics, and phrase and paragraph length, so that content would be readable regardless of screen size. For example, quiz questions had to be formatted so that the user would always see the question and all the possible responses without having to scroll down or across the screen.
- *Interactive glossary:* Biosafety is fraught with technical terminology, including words such as "confinement," which have a plain-English definition that is subtly different from its more technological definition when used in a biosafety context. The eLearning platform was therefore designed to incorporate a readily-accessible drop-down glossary into each module, which prompts the user when a new vocabulary item is encountered for the first time, and then would serve as a compendium of all vocabulary items associated across the portfolio, to which the user can refer at any time.
- *Exercises and intermediate tests:* Biosafety concepts are challenging to teach and to learn, primarily because many of the high-level concepts are grounded in one or more fundamental concepts. Any misunderstanding of these basic concepts, such as the meaning of *hazard*, may make it impossible for a user to then understand related concepts, such as *risk assessment* and *risk management*, which incorporate and rely on a clear understanding of *hazard*. The eLearning platform was therefore designed to enable the portfolio administrator to assess the degree of user understanding throughout the module, with the aid of short exercises and intermediate tests, as this helps to verify that the user understands all the fundamental concepts before then advancing further into the module with presentations of more complex concepts. Exercises and quizzes can also provide helpful feedback to the user, and even direct the user to review particular sections of the module in order to clarify and resolve any misunderstanding before delving further into the teaching.
- *Final exam:* To effectively verify that each user has mastered the key concepts of the module, the eLearning platform enables the content developer and/or host institution to include a final exam, which the user must complete and pass. Currently, the final exam (and any intermediate tests) of each module comprises a number of several types of questions randomly extracted from a much larger large database of questions. These question formats include: true/false, multiple-choice with only one correct answer, multiple-choice with more than one correct answer, ranking questions, and questions using an interactive diagram; and more question formats are under development. Of note, the options from which the user must select the correct answer(s) are randomly displayed each time the question is extracted from the main database. For example, an answer appearing as option A when first extracted, will randomly appear as option A, B, C, or D upon next extraction. In this way, users even when sitting alongside one another and undertaking

the exam at the same time, are not presented with the same question set, nor with the answers presented in the same order. For intermediate test only, once the user has completed the questions and received a final grade, the platform enables the user to go back through questions that were not answered correctly, to help them identify topics to review.

The platform can offer learning environments with differing pass rates (of the modules themselves, as well as for the portfolio overall), as well as differing options for re-sitting examinations, tailored to each hosting institution's requirements. To elaborate, each individual hosting institution can dictate: the number of questions from the database to be included in each test and exam, the pass rate for each, the pre-determined waiting period before any exam re-sit, and also the number of times the exams can be re-sat.

Together, these design features allow the creation of sophisticated and rigorous modules, comprising a variety of appropriate media to enhance learning. Previous research has identified a lack of compulsion to engage with online learning material as a potential obstacle to eLearning experiences, given the lack of a formal framework or timetable to which users are accountable (Reid et al., 2016). The ICGEB eLearning platform minimizes the likelihood of such a passive user experience, by incorporating exercises, quizzes, tests, and exam questions to check learning, provide feedback, and increase user engagement.

Design Features to Enhance Course Administration

First and foremost, the eLearning platform was designed to be easy for institutions to adopt. Because the platform is hosted remotely, there are no direct hosting costs or IT staff needs for the institution. Access to the platform, both for users and administrators, is provided through password-protected accounts. The administrator's account provides several functions to facilitate course administration: the authorisation of each user to enroll in the course; the oversight of each user's progress of the course, including user success in preliminary tests and the final exam; the ability to assign modules to users in a pre-determined order, and; the ability to impose deadlines by which all of the users will have completed each module.

Users can quickly and easily track their progress through the course using a personalized "dashboard," which highlights the overall percentage completion (macro-level) of each assigned module as a circular graph, for example a pie chart or gauge. This is the same when monitoring their progress at the micro-level, i.e., for each chapter in the module, as each of these also has its own gauge to demonstrate the percentage completion. In addition, the dashboard provides user access to external resources and required readings, along with additional resources devised as supplementary module content, and the course compendium glossary.

DEVELOPING ELEARNING MATERIALS BASED ON THE CURRICULUM

Once the context of the eLearning, i.e., curriculum structure with key learning goals and the design features of the platform were established, a process was put in place to deliver the actual content. In order to allow maximum flexibility, each module was planned to function both on its own and as a part of the larger course curriculum. For each module, at least two experts, internationally-recognized in providing biosafety training, were selected as primary content developers. The first phase consisted of exploratory briefings and exchanges amongst the developers (both content and IT) on the organization and content of the module, and an initial "storyboard" drafted to reflect all of the major elements to be included—this storyboard was continually being updated as the specific content was elaborated and organized. Modules were further divided into chapters and smaller units suitable for eLearning sessions, and especially to facilitate online streaming in narrow-bandwidth geographic areas. Working with smaller units also assisted in interspersing the module content with videos, reading materials, exercises, and other components that allow a user to work at their own pace.

The first phase provided a first level of critical review of content. It would later also result in a diversity of experts presenting the content, making it more engaging to the user. Although the experts had highly-recognized experience on the specific topics and therefore were more than capable of making in-depth presentations, the adaptation of the materials for the purposes of eLearning was an ever-present challenge. Although a general rule when presenting PowerPoint slides is to not use overly-long sentences, the number of words to eventually be displayed on the screen in the eLearning context had to be reduced even more. For complex scientific concepts and legal texts in particular, this was a difficult task, as quotations have to be complete and correct. In such instances, experts were advised to use, as much as possible, complimentary documents, rather than trying to force all of the information into a recorded video. This is one of the aspects where a good exchange amongst the content and IT developers is essential. While the IT developers need to understand the overall objectives of the course, experts need to understand: the capabilities of the platform tools and IT developers (especially in the field of graphics and animation); the limitations of a computer-based presentation, and; a "common language" with the IT developers, so that ambiguities are minimized and everyone understands how the overall objectives are to be achieved collectively.

Initial PowerPoint presentations prepared by the experts were then transferred to, and revised in, eLearning-ready templates. The resulting eLearning-ready presentations included all of the necessary technical indications such as animation, timings, and suggestions for specific graphics. Whenever an external third-party source was required (document, video, picture, etc.), appropriate authorisation was obtained, even if the source was freely-available on the Internet. The eLearning-ready presentations were verified once more by the experts who had developed the materials, and acted as a second level of quality check. This check was also important in order to ensure consistency between the different modules. For example, concepts such as *biosafety* or *biorisk* may have slightly different meanings in different contexts and, if not specifically mentioned, may confuse the user.

Video content was recorded in a professional study, by a cameraman and sound technician. One key observation made

by the experts was that recording a video is a very different experience to providing a lecture for a live audience. In spite of the experience of all experts with live audiences, the professional insights of the recording crew were required to allow smooth transitions between slides. With the scenario clearly spelled out in the eLearning storyboard, including the placement of exercises and preliminary tests, the recordings were then tailored to facilitate post-editing. Also, the crew ensured that the atmosphere allowed a natural and relaxed recording, in spite of the many hours confinement to a specific fixed spot under hot lights. At the end of the recording, all results were reviewed with the IT developers and final arrangements were cross-checked for additional materials, glossary, exercises, etc.

The remaining steps were the production of a module "Alpha" version, which was a compendium of all of the integrated recorded and non-recorded information, that was subjected to a third verification and edits by the content and IT developers, before the resulting "Beta" version was subjected to peer-review by external biosafety and biosecurity experts in order to help identify any inaccuracies, contradictions, omissions, or inconsistencies in the content, along with any software faults, bugs or difficulties in access/use. Their comments were addressed by the content and IT developers, eventually leading to the final "production" version of the module.

DISCUSSIONS AND CONCLUSIONS

From the perspective of content developers, key take-home messages from the eLearning experience include:

- Learning objectives should be clearly elaborated at the outset to ensure that each module covers all of the intended subject material, and that it interconnects within the overall curriculum.
- Experts should be briefed on the possibilities and limitations of the eLearning platform, in order to prepare content that provides the best learning experience.
- Experts need to be mentally prepared for a non-audience presentation, overcoming the absence of direct contact and feedback from a live audience. The timing of the appearance of images and text is very different.
- Experts must be made aware of the diversity of materials that can be used and should select the best suited format, e.g., background documents for longer texts.
- Consistency is essential and tools, such as a common glossary, should be compiled for all modules and shared by all experts.
- The development of a "common language" and good exchanges between the content and IT developers can result in magic! Discussing the options and clarifying the intentions of a slide may result in powerful images that will better attract the attention of the user.

The current portfolio of biosafety modules offers great promise in the development of autonomous and enduring biosafety systems that are relevant, useful, and resilient. The design of this portfolio encompasses not only GMOs, but also matters related to biosecurity, public health, natural resource management, biocontrol, and bioremediation. In fact, the breadth of coverage offered the portfolio was of especial focus, so that it appeals to users with different access points in technology regulation, including the regulated community, the regulators, and wider stakeholders with roles in regulatory decision-making. The most obvious benefits from utilizing eLearning in biosafety training include:

- **Flexible approach**—Provides hosting institutions as well as users with the flexibility of time and place of delivery.
- **Comprehensive coverage**—Enhances the efficacy of knowledge and qualifications via an ease of access to a huge amount of information.
- **Cost-effectiveness**—No need for training recipients (i.e., users) to travel to training venues. Also offers learning opportunities to a maximum number of users, without the need for education premises and facilities.
- **User-friendly**—Takes into consideration user's individual learning styles and paces.
- **Sustainable**—Helps compensate for any local scarcities of experts, allowing hosting institutions to take charge of local training needs, at minimal expense, and staff time investment.

Efforts are underway to not only offer the current portfolio of eLearning modules in courses to additional hosting institutions around the world, but also to extend the portfolio (and thus possible courses) through the development of modules covering complementary topics and approaches, especially in the field of government oversight of technological applications in biology-based sectors. In addition, investigations are being made into the availability of additional IT tools and accessories to help enhance the user educational experience, especially at the local level, and to continue tailoring the offered training to the future needs and potentials of the target user communities and hosting institutions.

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

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A General Safety Assessment for Purified Food Ingredients Derived From Biotechnology Crops: Case Study of Brazilian Sugar and Beverages Produced From Insect-Protected Sugarcane

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Insect-protected sugarcane that expresses Cry1Ab has been developed in Brazil. Analysis of trade information has shown that effectively all the sugarcane-derived Brazilian exports are raw or refined sugar and ethanol. The fact that raw and refined sugar are highly purified food ingredients, with no detectable transgenic protein, provides an interesting case study of a generalized safety assessment approach. In this study, both the theoretical protein intakes and safety assessments of Cry1Ab, Cry1Ac, NPTII, and Bar proteins used in insect-protected biotechnology crops were examined. The potential consumption of these proteins was examined using local market research data of average added sugar intakes in eight diverse and representative Brazilian raw and refined sugar export markets (Brazil, Canada, China, Indonesia, India, Japan, Russia, and the USA). The average sugar intakes, which ranged from 5.1 g of added sugar/person/day (India) to 126 g sugar/p/day (USA) were used to calculated possible human exposure. The theoretical protein intake estimates were carried out in the "Worst-case" scenario, assumed that 1 μg of newly-expressed protein is detected/g of raw or refined sugar; and the "Reasonable-case" scenario assumed 1 ng protein/g sugar. The "Worst-case" scenario was based on results of detailed studies of sugarcane processing in Brazil that showed that refined sugar contains less than 1 μg of total plant protein /g refined sugar. The "Reasonable-case" scenario was based on assumption that the expression levels in stalk of newly-expressed proteins were less than 0.1% of total stalk protein. Using these calculated protein intake values from the consumption of sugar, along with the accepted NOAEL levels of the four representative proteins we concluded that safety margins for the "Worst-case" scenario ranged from 6.9×10^5 to 5.9×10^7 and for the "Reasonable-case" scenario ranged from 6.9×10^8 to 5.9×10^{10}. These safety margins are very

high due to the extremely low possible exposures and the high NOAELs for these non-toxic proteins. This generalized approach to the safety assessment of highly purified food ingredients like sugar illustrates that sugar processed from Brazilian GM varieties are safe for consumption in representative markets globally.

Keywords: sugar, highly purified substances, Cry1Ab, Cry1Ac, NPTII, bar, *Saccharum*, sugarcane

INTRODUCTION

In Brazil alone, sugarcane borer, a lepidoptera that feeds on sugar cane plants, costs the sugarcane industry a billion US dollars in crop damage and processing costs yearly. The propagation of the first biotechnology-derived Cry1Ab-expressing sugarcane variety was approved and launched in Brazil in late 2017. Cry1Ab and Cry1Ac proteins target receptors found only in lepidoptera, causing selective toxicity. These proteins have proved very effective, as their toxicity is specific, and can thus be used to target specific lepidoptera pests. Given these attributes, research has shown that Cry1Ab and Cry1Ac are useful in sugarcane agronomy and production, benefits including improved plant protection and reduced pesticide use. The Brazilian sugarcane processing industry is highly integrated and focused on the production of ethanol (for primarily domestic energy markets) and sugar for domestic and export markets. Careful analysis of the foreign trade information regarding Brazilian sugarcane-derived products exported to key markets show that the article of commerce relating to human food is sugar, either raw sugar, or refined sugar. The by-products of sugarcane processing, such as the bagasse (fiber) and molasses are recycled within industrial processing employed by Brazilian mills and are not exported in any appreciable amounts globally. This trade situation, and the highly refined nature of either raw or refined sugar, creates the possibility to consider a broad-based approach to establishing the safety of many widely-used proteins based on sound scientific and policy foundation. A key aspect of sugarcane processing and the production of raw and refined sugar from sugarcane, involves the extensive processing with heat and pH adjustment that effectively removes all detectable DNA and proteins from raw and refined sugars (Cheavegatti-Gianotto et al., 2011).

Various sugarcane processing studies done by Cullis et al. (2014) who examined total DNA and protein loss, by Cheavegatti-Gianotto et al. (2018) who studied Rubisco and Cry1Ab loss in Bt sugarcane and by Joyce et al. (2013) have established that sugarcane processing effectively eliminates detectable DNA and protein from raw or refined sugar. Other studies measuring specific transgene DNA or protein in sugar beets have also shown that raw or refined sugar produced from sugar beets do not contain detectable DNA or protein. In a comprehensive study, Cullis et al. (2014) established that total protein levels were below 1 microgram per-gram of refined sugar; however, the theoretical levels of newly-expressed GM protein would be orders of magnitude below this value, depending ultimately on the level of protein expression as a percent of total protein content. Since the presence of DNA and protein in raw and refined sugar examined by Cullis et al. (2014) and Cheavegatti-Gianotto et al. (2018) were below the limit of quantification, even using highly sensitive methods, any possible human dietary exposure to these proteins would be extremely low.

In this study, the safety of four commonly used proteins in GM crops were examined; the Cry1Ab and Cry1Ac proteins used in crops to control lepidoptera pests, NPTII, a commonly used selectable marker protein and Bar, an herbicide-tolerance trait also used as a selectable marker. These proteins have been studied extensively and approved widely by regulatory agencies worldwide. Results of acute toxicology studies have established No Observed Adverse Effect Levels (NOAELs) and significant confirmatory data exists from a variety of subchronic toxicity and other studies. Digestibility studies *in vitro* have shown that these proteins are rapidly degraded in either mock gastric or intestinal fluids indicating that they are digested readily and not available for oral absorption. Using this wealth of information, it is possible to evaluate the safety of sugarcane products expressing these proteins using a first-principles approach that incorporated the extremely low theoretical amounts in raw or refined sugar, the known intakes of added sugar in various representative export markets and the established safety of these proteins. For these four proteins, the country specific added sugar average intake values were examined using two scenarios of possible presence in raw/refined sugar. This exposure-driven approach established extremely large safety margins for these proteins and provides a general approach to safety assessment of other highly processed food and feed ingredient products derived from GM crops.

METHODS

Estimation of the Theoretical Concentration of the Newly Expressed Protein in Raw and Refined Sugar: Worst-Case and Reasonable-Case Assumptions

Using the results of Cullis et al. (2014) and Cheavegatti-Gianotto et al. (2018) and Reasonable assumptions regarding the level of expression of Cry1Ac, Cry1Ab, NPTII, and Bar proteins in stalk, two scenarios were developed regarding possible presence of these proteins in raw or refined sugar. It is important to note that the varieties of sugarcane that may contain the four proteins examined are/will be developed for cultivation in Brazil (Center-South and Northeast) and will not be commercialized elsewhere. Therefore, the potential exposure of consumers outside of Brazil will occur via the processing and export of either raw or refined sugar.

The first Scenario assumes that the concentration estimate of each of the four newly-expressed proteins is 1 μg newly-expressed protein/g refined sugar. This "Worst-case" estimate clearly is a significant overestimate of the actual concentration of each newly-expressed protein/g raw or refined sugar. Cullis et al. (2014) examined the loss of TOTAL sugarcane stalk protein during the processing of sugar in Brazil. Using sensitive detection methods, they found that protein was not detectable using these methods at < 1 μg/g refined sugar. Consequently, Scenario A is based on this detection level and assumes that all the protein in the sugar is that specific protein (e.g., Cry1Ab). The second scenario, described herein as "Reasonable-case," assumes that the concentration of the specific newly-expressed transgenic protein represent 0.1% of total stalk protein, a more Reasonable scenario given the experiences to date with GM sugarcane. Therefore, for the "Reasonable-case" scenario, it is assumed that the actual concentrations of the newly-expressed proteins are only 0.1% of the 1 μg/g refined sugar value reported by Cullis et al. (2014) reported for total protein. In the case of CTC's Bt sugarcane recently approved in Brazil, the actual expression levels of both Cry1Ab and NPTII were much lower than 0.1% of total stalk protein, both of which were below the limit of quantification of 235 and 34 ng/g stalk tissue, respectively (Cheavegatti-Gianotto et al., 2018).

In conclusion, the assumed concentration of newly-expressed protein in refined sugar was 1 ppm for the "Worst-case" and 1 ppb for the "Reasonable-case" scenarios. It is noteworthy that concentrations in sugar consumed chronically in the various eight country markets will be diluted substantially by non-Brazilian sourced raw or refined sugar; for example, the contribution of imported sugar from Brazil to the amount of added sugar consumed in the Canada and the US is estimated at 11% (FAO/WHO, 2000).

Estimation of Added Sugar Mean Intakes and Derived "Worst-Case" and "Reasonable-Case" Intakes of Newly Expressed Proteins in Eight Representative Markets of Exported Brazilian Raw and Refined Sugar

It is not simple to compare similar data regarding added sugar consumption across various countries as differing methods are used in government-funded surveys of food intakes and composition. In our study, data collected in 2015 by Euromonitor International was used (Ferdman, 2015). Euromonitor International nutrition methodologies assess the probable mean intakes/person/day of eight nutrients: energy, protein, carbohydrates, sugar, fat, saturated fat, fiber and salt. The examined packaged foods and fresh foods and beverages based on nutrient content information and intake information in 54 countries globally. This study approach using consistent market research methodologies was used in our case study because it represented a similar approach in all countries examined herein.

The mean sugar intakes reported by Euromonitor International across various countries were typically higher than those found in published research. For example, research performed by the Canadian Sugar Institute, found average sugar intake to be 50 (g/p/d) compared to the 89.1(g/p/d) value found by Euromonitor International marketing research. Marketing research values were also higher than the USDA NHANES values in the United States, the former being 126.4 (g/p/d) compared with the NHANES data of 82 (g/p/d). The differences in intake survey-based results, like those described by the Canadian Sugar Institute and USDA NHANES, compared with the Euromonitor International results, are likely due to differences in methodology. The Euromonitor International data reports food disappearance vs. food consumption as estimated by dietary surveys. As a result, the Euromonitor International results shown in **Tables 4**, **5** are likely overestimates of actual ingredient intake. Therefore, for consistency across geographies, these Euromonitor International "overestimate" ingredient intake results were preferred and used in both our "worst case" and "reasonable case" estimates of sugar intake.

All estimated "Worst-case" and "Reasonable-case" estimates of theoretical protein intake for the four subject Cry1Ab, Cry1Ac, NPTII, and Bar proteins were calculated using the Euromonitor International marketing research mean added sugar values and assumed concentrations in sugar of 1 ppm and 1 ppb, respectively. Using these estimated intakes (**Tables 4**, **5**), and the internationally accepted NOAELs for the Cry1Ab, Cry1Ac, NPTII, and Bar proteins, it was possible to calculate safety margins as the NOAEL for each protein divided by estimated average protein intakes from added sugar in the eight countries. In order to evaluate the safety of these proteins, it is necessary to summarize the toxicology and related safety data for these individual four proteins.

Summary of Toxicology and Safety Information on the Four Newly-Expressed Proteins in GM Sugarcane: Published Literature and NOAEL Values

The four specific proteins selected in this assessment were chosen because both the Cry1Ab and Cry1Ac proteins are useful in the management of sugarcane borer in Brazilian sugarcane production and the NPTII and Bar proteins are widely used as selectable markers. Each of these proteins have both been widely used in other agricultural biotechnology crops and extensively studied and reviewed by regulatory agencies worldwide. As shown in **Table 2**, the US Environmental Protection Agency (EPA) has reviewed the safety of these pesticidal (Cry1Ab and Cry1Ac) and inert (NPTII and Bar) proteins. EPA concluded, based on a variety of the data presented below, that these proteins were safe for their potential use in all crops, including possible use in sugarcane. This conclusion was based on considerations like history of safe use (in bacterial pesticidal sprays used in organic agriculture), animal toxicology studies, studies on the digestibility of the proteins and bioinformatics studies for potential allergens or toxins. The safety assessments conclusions referenced in this manuscript were written by EPA and European Food Safety Authority (EFSA) and based on published literature and product-specific submissions by Bayer, Monsanto, Syngenta and other companies. The studies and assessments follow the

Codex Alimentarius "Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants" (2003). These assessments included the following information described below: acute and subchronic toxicology studies, *in vitro* digestibility and heat lability studies, and bioinformatics assessments of potential allergenicity and toxigenicity. The key results for each newly-expressed protein are summarized below that led to the determination of the individual NOAEL for each protein.

Acute Toxicology Studies

Table 1 shows the results of Acute and Subchronic toxicology studies for Cry1Ac, Cry1Ab, NPTII, and Bar. The studies were primarily conducted using mice administered the protein either by acute gavage or in the diet; the exceptions being that an acute toxicity study on Bar protein was done using intravenous dosing and a subchronic study that was conducted in mice. The goal of these studies was to determine the NOAEL for the specific tested substances. There were no adverse effects of any protein at the highest dose tested and therefore the highest dose tested is the NOAEL. Often the highest dose tested was due to physical chemical constraints like the solubility of the protein in the injection solution. The results (**Table 1**) shows that the oral NOAEL for Cry1Ab and Cry1Ac were $\geq$ 4,000 and 1,460 mg/kg bw, respectively. Similarly, the oral NOAEL for NPTII was found to be $\geq$ 5,000 mg/kg bw. The highest dose tested was either limited by the solubility of the protein in the dosage formulation or the accepted maximum dose tested in 5,000 mg/kg bw acute toxicity limit test. These results establish that these proteins are essentially non-toxic. By comparison to the studies with Cry1Ab, Cry1Ac, and NPTII proteins, the NOAEL of the Bar protein was determined following intravenous dosing and not oral dosing. There were no effects observed after 10 mg/kg bw iv dosing, the highest dose administered. Given that the Bar protein was rapidly degraded in simulated gastric and intestinal juices incubations (within seconds to minutes), it is reasonable to expect that the NOAEL for Bar protein orally is several orders of magnitude higher than the iv NOAEL dosing value of 10 mg/kg bw. Regardless, as a result of this difference in route of administration and dosing limitation, the NOAEL for Bar is the lowest amongst these proteins.

Subchronic Toxicology

Along with acute toxicology studies, focused on finding the NOAELs for Cry1Ac, Cry1Ab, NPTII, and Bar, subchronic toxicology studies were also performed in an effort to study possible longer-term effects of the proteins. In these studies, the proteins were administered orally, either by gavage or in the diet (as a constituent of GM grain used to formulate the diets), for 90 days. As **Table 1** shows no adverse effects observed at the highest doses administered for these proteins. In addition to the results of these studies, numerous other studies have been conducted and report on the grain/processed fraction produced from Cry1Ab and Cry1Ac-expressing biotechnology crops. These studies confirmed that there were no adverse effects at the highest doses tested. As a consequence of the entire weight of the evidence regarding the safety of these protein, toxicologists and regulators have concluded that the most appropriate NOAELs are those determined by the acute toxicology studies noted above.

In Vitro Digestion Stability Tests

Digestive stability testing was performed in a mock *in vitro* digestive environment simulating both a gastric and intestinal fluid. The goal of these tests is to estimate the rate of protein degradation or denaturation in the human digestive systems. Proteins that are rapidly denatured (by low pH) and enzymatically digested (by intestinal enzymes) have a lower probability of producing either toxicity since digestion products are common peptides or dietary amino acids or allergenicity because the protein is not present to elicit antibody production

TABLE 1 | Toxicology study results for Cry1Ab, Cry1Ac, NPTII, and bar proteins.

	Cry1Ab	Cry1Ac	NPTII	Bar
Acute Toxicology	Protein was orally administered to mice. Five male and five female mice were given doses up to 4,000 mg/kg bw. No effects were observed.	Protein was orally administered to 8–10 week mice. Mice were given a single dose at 1,280–1,290 mg/kg bw. No effects were observed.	Protein was orally administered to 10 males and 10 female mice. Doses were administered 100, 1,000, and 5,000 mg/kg bw. No effects were observed.	Protein was intravenously administered to mice at doses of 1 and 10 mg/kg bw. No effects were observed.
Subchronic Toxicology	Rice containing Cry1Ab was feed to mice over a 13 week period. Rodents were fed rice at three separate protein concentrations (17.5, 35, and 70 %). No effects were observed.	Cry1Ac containing diets were administered to mice over a 13 weeks period. Rodents were fed maize at three separate protein concentrations (12.5, 25, 50%). No detectable Cry1Ac-M protein was found in the serum of rats after feeding diets containing GM maize for 3 months. The results demonstrated that BT-38 maize is as safe as conventional non-GM maize.	NPTII containing diets were administered to mice over a 13 weeks period. Rodents were fed maize at three separate protein concentrations (12.5, 25, 50%). No detectable NPTII protein was found in the serum of rats after feeding diets containing GM maize for 3 months. No effects were observed. Studies done on BT-38 maize.	Protein was incorporated in the diet of mice for 90 days at levels of 0, 5, and 50 g/kg diet, this was done over a 2 week period. Corresponding to 7.6 and 7.9 mg/kg BW/day for males and females. No effects were observed.
NOAEL (mg/kg bw)	>4,000	>1,460	>5,000	>10

or allergic reactions. Cry1Ab, Cry1Ac, NPTII, and Bar are all degraded quickly in these *in vitro* mock digestive solutions, making them significantly less likely to exhibit local or systemic toxicity or allergenicity, as protein exposure is transient. The conclusions from the results of these tests, as concluded by EPA, for the Cry1Ab, Cry1Ac, NPTII, and Bar proteins, are provided below.

Cry1Ab

"The *in vitro* digestion assays confirm that the protein is being broken down in the presence of typical digestive fluids and is not unusually persistent in the digestive system. All were degraded in gastric fluid in 0–7 min" (Kough et al., 2010).

Cry1Ac

"The Cry1Ac protein was digested within 30 s in simulated gastric fluid containing pepsin. Small peptides remaining following gastric simulated digestion were completely degraded to amino acid residues in SIF (simulated intestinal fluid) upon contact" (Kough et al., 2010).

NPTII

"NPTII degrades extremely readily in SGF (simulated gastric fluid). No NPTII protein was detected, by western blot analysis, at the first incubation time point of 10 s. In SIF (simulated intestinal fluid), NPTII also degrades readily with 50% degradation occurring after 2–5 min of incubation at 37°C." (Fuchs et al., 1993)

Bar

"Bar proteins were degraded very rapidly and completely in the SGF (simulated gastric fluid) (pH 2) or SIF (simulated intestinal fluid) (pH 7.5), within few seconds of incubation, in the presence of pepsin or pancreatin, respectively. … In the SIF (simulated intestinal fluid) assay, the complete degradation of remaining 7-kDa fragments was achieved within 5 min rather than a few seconds." (Hérouet et al., 2004).

EPA Exemptions From Tolerance

After having reviewed the extensive database supporting the safety of these four proteins separately, EPA concluded that the proteins have safety profiles that permit them to be exempted from the need for tolerances in food or feed in the United States. The EPA issued these exemptions from tolerances for the Cry1Ab, Cry1Ac, NPTII, and Bar proteins for use in any crop based on several factors including the very high NOAELs. These EPA exemptions are listed below in **Table 2**.

International Approvals for the Newly-Expressed Proteins

The safety of these proteins has also been widely reviewed by regulatory agencies globally. As shown in **Table 3**, products containing the newly-expressed proteins Cry1Ac, Cry1Ab, NPTII, and Bar have been approved for consumption in many countries worldwide. Given the breadth of biotechnology crops utilizing these proteins, these approvals further confirm the conclusions drawn by the US EPA and the European Union EFSA regarding the safety of the food and feed produced from these crops.

Calculated Safety Margins

Based on the "Worst-case" and "Reasonable-case" theoretical protein exposure values from added sugar and the NOAEL values for each of the four proteins, safety margins for the Cry1Ab, Cry1Ac, NPTII, and Bar proteins were calculated. These safety margin values were calculated by dividing the protein-specific NOAELs, expressed in μg/kg bw/d, by the mean exposure estimate also expressed as μg/kg bw/day.

RESULTS

The mean added sugar intakes in the eight selected countries that are markets for Brazilian-produced sugar varied significantly, probably as a result of dietary preferences and socio-economic factors. Calculated safety margins for the four newly-expressed proteins for the "Worst-case" and "Reasonable-case" exposure scenarios are provided in **Tables 4**, **5**, respectively. Mean added sugar consumptions for the eight sample countries are also shown in **Tables 4**, **5**. The tables show India, China and Indonesia to be the low consumers, Russia and Brazil to be intermediate consumers, and Japan, Canada and the United States to be the higher consumers. It appears that dietary preferences and socio-economic differences between the countries may contribute this broad distribution in average added sugar intakes. Regardless, the theoretical intakes at the "Worst-case" and "Reasonable-case" scenarios are related directly with the sugar intake figures: theoretical protein intakes were lower in India, China and Indonesia, intermediate in Russia and Brazil, and highest in Japan, Canada and the United States.

Table 4 also shows the calculated safety margins for each newly-expressed protein using the "Worst-case" scenario exposure in the eight selected countries. The "Worst-case" scenario safety margins in the eight countries for the three proteins for which the NOAELs were established by oral dosing (i.e., Cry1Ab, Cry1Ac, and NPTII) ranged from 6.9×10^5 to 4.7×10^7; the safety margins for the Bar protein were lower as a result of the lower NOAEL value at the highest dose tested following intravenous dosing. The resulting Bar protein safety margins were lower and ranged from 4.7×10^3 to 1.2×10^5. The lower safety margins for the Bar protein was based on the fact that the protein was administered intravenously at 10 mg/kg bw; the actual oral NOAEL for Bar would undoubtedly be orders of magnitude higher given the rapid degradation in both simulated gastric and intestinal fluids. Nonetheless, the safety margins for all four proteins, using the "Worst-case" scenario, were at least 10^3, well above those considered by toxicologists and regulators globally to establish dietary safety.

Table 5 shows the calculated safety margins for each newly-expressed protein using the "Reasonable-case" scenario in the eight selected countries. The "Reasonable-case" scenario safety margins in the eight countries for the three proteins for which the NOAELS were established by oral dosing (i.e., Cry1Ab, Cry1Ac, and NPTII) ranged from 6.9×10^8 to 4.7×10^{10}; the safety margins for the Bar protein were lower and ranged from 4.7

TABLE 2 | EPA exemptions from tolerances for Cry1Ab, Cry1Ac, NPTII, and Bar.

Cry1Ab (*Bacillus thuringiensis*)	Cry1Ac (*Bacillus thuringiensis*)	Bar (Phosphinothricin Acetyltransferase (PAT) enzyme)	NPTII (neomycin phosphotransferase II)
"Residues of *Bacillus thuringiensis* Cry1Ab protein in all plants are exempt from the requirement of a tolerance when used as plant-incorporated protectants in all food commodities." → **40 CFR 174. 511**	"Residues of *Bacillus thuringiensis* Cry1Ac protein in all plants are exempt from the requirement of a tolerance when used as plant-incorporated protectants in all food commodities." → **40 CFR 174.510**	"Residues of the Phosphinothricin Acetyltransferase (PAT) enzyme are exempt from the requirement of a tolerance when used as plant-incorporated protectant inert ingredients in all food commodities." → **40 CFR 174.522**	"Residues of the neomycin phosphotransferase II (NPTII) enzyme are exempted from the requirement of a tolerance in all food commodities when used as a plant-incorporated protectant inert ingredient." → **40 CFR 174.521**

TABLE 3 | Country and product approvals for Cry1Ab, Cry1Ab, NPTII, and bar.

Protein	Number of countries to approve	Number of events approved	Crops approved in
Cry1Ac	54	38	Cotton, Eggplant, Maize, Poplar, Rice, Soybean, Tomato, Sugarcane
Cry1Ab	55	57	Alfalfa, Apple, Canola, Chicory, Cotton, Eggplant, Eucalyptus, Flax, Maize, Melon, Papaya, Plum, Popular, Potato, Squash, Sugar beet, Sugarcane, Sugar Beet, Tobacco, Tomato
NPTII	57	121	Alfalfa, Apple, Canola, Chicory, Cotton, Eggplant, Eucalyptus, Flax, Maize, Melon, Papaya, Plum, Popular, Potato, Squash, Sugar beet, Sugarcane, Sugar Beet, Tobacco, Tomato
Bar	47	55	Canola, Chicory, Cotton, Maize, Rice, Soybean, Sugarcane

$\times 10^6$ to 1.2×10^8. The safety margins for the "Reasonable-case" scenario, were at least 10^6, well above those considered by toxicologists and regulators globally to establish the safety.

DISCUSSION

CTNBio, the Brazilian government regulatory authority involved in the review and approval of biotechnology-derived products, recently approved a Cry1Ab-expressing sugarcane plant for cultivation in Brazil (CTNBio, 2017). The CTNBio assessment considered a wide range of data on the Cry1Ab-expressing sugarcane variety including agronomic and phenotypic studies, non-target organism studies, molecular and protein characterization, protein expression in sugarcane tissues, effects of sugarcane processing on DNA and protein in raw and refined sugar, and product food and feed safety assessment. Based on these assessments, CTNBio approved the product for cultivation in the Center-South growing region in Brazil. With this approval, CTC has started controlled bulk-up/propagation field activities and commercial scale sugar production will occur in 2020.

Brazil is a major supplier of raw or refined sugar globally. Analysis of the export data over the last 5 years of sugarcane-derived products from Brazil show that the vast majority of exported sugarcane-derived products is raw or refined sugar; consequently, the major articles of commerce are highly purified ingredients. Brazilian foreign trade exports to the top 20 sugar markets, which include most of the eight countries studied herein, shows that virtually all of sugarcane-derived Brazilian exports are raw or refined sugar. A trace amount of exports of distilled alcoholic beverages does occur in some countries but the fermentation and distillation process would certainly remove proteins. The exported raw sugar is at least 97% pure sucrose (OECD, 2011). In many countries, food regulations require that raw sugar for human consumption be further refined, to avoid contamination which may occurs during transport of commodities, and the final purity of refined sucrose is over 99.7 % (OECD, 2011).

As a result of the high temperatures, pH adjustments and sucrose crystallization conditions produced in the processing of sugarcane stalk to raw and refined sugar it is not surprising that sugar does not contain detectable quantities of DNA or protein. Several investigators have evaluated this processing loss of DNA and protein, examining the loss of both endogenous and exogenous DNA or protein including total protein and DNA and Rubisco DNA and protein (Cullis et al., 2014; Cheavegatti-Gianotto et al., 2018). This conclusion is further substantiated by Cheavegatti-Gianotto et al. (2018) including data on the lack of detection of Cry1Ab in processing fractions including clarified juice, raw and refined sugar produced from Bt sugarcane in Brazil. Similar findings have been reported for loss of newly-expressed proteins in GM sugarcane (Joyce et al., 2013) and sugar beets (Klein et al., 1998; Oguchi et al., 2009).

The highly purified nature of raw and refined sugar allowed for the development of a generalized safety assessment Case Study given the low detection limits and range of sugar consumption levels worldwide. The seven export countries researched in this studied, in addition to Brazil itself, were chosen because they are chief importers of Brazilian sugar and, more importantly, because they all have established regulatory agencies that review biotechnology-derived crops and derived food ingredients. The Cry proteins researched in this study, both of which have been proven extremely effective against sugarcane borer, were chosen as they have been widely approved around the world including in the eight countries researched in this study. The selectable marker proteins researched, NPTII and Bar, were

TABLE 4 | Safety margins at "Worst-case" Protein exposure in eight countries.

Country	Marketing research mean added sugar consumption (g/p/d)	Worst-case sugar intake (mg/kg bw/d)	Maximum protein exposure (μg/kg bw/d)	Calculated safety margins Cry1Ab	Calculated safety margins Cry1Ac	Calculated safety margins NPTII	Calculated safety margins bar
Brazil (a)	47.6	0.79	0.79	5.1×10^6	1.8×10^6	6.3×10^6	1.3×10^4
Canada (b)	89.1	1.49	1.49	2.7×10^6	9.8×10^5	3.4×10^6	6.7×10^4
China (c)	15.7	0.26	0.26	1.5×10^7	5.6×10^6	1.9×10^7	3.8×10^4
Indonesia (d)	15.2	0.25	0.25	1.6×10^7	5.8×10^6	2.0×10^7	4.0×10^4
India (e)	5.1	0.085	0.085	4.7×10^7	1.7×10^7	5.9×10^7	1.2×10^5
Japan (f)	56.7	0.95	0.95	4.2×10^6	1.5×10^6	5.3×10^6	1.1×10^4
Russia (g)	20.0	0.33	0.33	1.2×10^7	4.4×10^6	1.5×10^7	3.0×10^4
US (h)	126.4	2.11	2.11	1.9×10^6	6.9×10^5	2.4×10^6	4.7×10^3

TABLE 5 | Safety margins at "Reasonable-case" protein exposure in eight countries.

Country	Marketing research sugar consumption (g/p/d)	Worst-case sugar intake sugar consumption (mg/kg bw/d)	Reasonable protein theoretical exposure (μg/kg bw/d)	Calculated safety margins Cry1Ab	Calculated safety margins Cry1Ac	Calculated safety margins NPTII	Calculated safety margins bar
Brazil (a)	47.6	0.79	0.00079	5.1×10^9	1.8×10^9	6.3×10^9	1.3×10^7
Canada (b)	89.1	1.49	0.00149	2.7×10^9	9.8×10^8	3.4×10^9	6.7×10^7
China (c)	15.7	0.26	0.00026	1.5×10^{10}	5.6×10^9	1.9×10^{10}	3.8×10^7
Indonesia (d)	15.2	0.25	0.00025	1.6×10^{10}	5.8×10^9	2.0×10^{10}	4.0×10^7
India (e)	5.1	0.085	0.000085	4.7×10^{10}	1.7×10^{10}	5.9×10^{10}	1.2×10^8
Japan (f)	56.7	0.95	0.00095	4.2×10^9	1.5×10^9	5.3×10^9	1.1×10^7
Russia (g)	20.0	0.33	0.00033	1.2×10^{10}	4.4×10^9	1.5×10^{10}	3.0×10^7
US (h)	126.4	2.11	0.00211	1.9×10^9	6.9×10^8	2.4×10^9	4.7×10^6

chosen as they were also approved in all eight of the sample countries.

Based on the marketing research data conducted by EuroMonitor International, we were capable of examining mean added sugar intake in the eight sample countries using similar methodologies. Several of these countries conduct nutritional intake surveys and analyses to examine nutritional trends and develop nutritional guidance; however, across-country comparisons, often using differing methodologies, are not amenable to side-by-side comparisons. Therefore, the marketing research approach used by Euromonitor International was used. The results of mean sugar intake data showed a large range with the lowest intakes occurring in developing economies (India, Indonesia, China and Brazil) and the higher intakes in more developed economies (United States, Canada and Japan).

The proteins examined in this study have been widely used in agricultural biotechnology products globally. The Cry1Ab and Cry1Ac proteins have been shown to be effective in managing lepidoptera pests in various crops including in sugarcane production in Brazil. The NOAELs, the highest dose tested that was not associated with any adverse effects in animals for these four proteins, are generally well established and accepted by regulatory agencies including the US EPA and the European Union EFSA. Safety margin results for Cry1Ab and Cry1Ac were very high for both scenarios. For Cry1Ac the "Worst-case" safety margins ranged from 6.9×10^5 to 1.7×10^7, while the "Reasonable-case" safety margins ranged from 6.9×10^8 to 1.7×10^{10} (see **Table 4**). For Cry1Ab the "Worst-case" safety margins ranged from 1.6×10^6 to 4.7×10^7, while the "Reasonable-case" safety margins ranged from 1.6×10^9 to 4.7×10^{10} (see **Table 5**). The immensity of the safety margins presented here are difficult to interpret in the abstract. Typically, safety margins of 100–500 is required for a new food ingredient added to food or beverage; in other words, the allowable daily intake of the ingredient is often determined as the lowest NOAEL divided by 100 (i.e., a 100 fold safety margin). The safety margins calculated under the "Worst-case" and "Reasonable-case" exposures for sugar are several orders of magnitude higher than those used by food toxicologists and regulators for other food ingredients. It is also possible to consider the extremely large safety margins for sugar in the context of the amount of sugar that would need to be consumed to reach the NOAEL values. For example, using Cry1Ac protein safety (1,460 mg/kg bw), a 60 kg person consuming refined sugar with a Cry1Ac concentration $< 1\,\mu g/g$ sugar would need to ingest 87.6 metric tons or 192,720 lbs of added sugar to theoretically exceed the NOAEL value.

The scenarios studied were chosen because they represent extreme overestimates. The "Worst-case" scenario assumed that all of the possible protein present at the Cullis et al. (2014) limit of quantification was the protein of interest—clearly a great

overestimation. Even the "Reasonable-case" scenario assumed that 0.1% of the total stalk protein was the protein of interest. In addition, these scenarios do not take into account two important sources of "dilution" of the raw and refined sugar produced from Brazilian biotechnology-derived varieties; the first is dilution within Brazil by non-GM derived sugar and the second is dilution in the local market (e.g., India, Japan, US). Brazilian varieties expressing Cry1Ab or Cry1Ac will be suited for specific growing regions. Given that plant propagation of new varieties is slow, taking 3 years to reach only one-two percent market share, it is reasonable that the proportion of Brazilian sugarcane that is biotechnology-derived and expressing Cry1Ab or Cry1Ac will be less than 20% for several years to come. The dilution of Brazilian produced sugar by other sources of sugar also significantly lowers the possible protein concentrations studied in the various scenarios and export countries. For example, in the case of the United States, only 1–2% of sugar consumed by Americans is produced in Brazil. This means, at least for the United States, the safety factor could be up to another two orders of magnitude higher. Again, the purpose of using the "Worst-case" and "Reasonable-case" scenarios were to provide tangible examples of the relationship between exposures and safety margins, especially for such highly purified ingredients. The overall conclusion of this study is that the possible exposures to these four newly expressed proteins are trivial compared with the known safety NOAELs for these proteins. These conclusions are valid regardless of the exact level of protein expression in the stalk. It is, of course, necessary to confirm, on a variety-by-variety basis, that the expressed proteins are identical to the proteins tested for the determination of NOAELs. Given the lack of detection of protein at low limits of detection, it should be possible to regulators worldwide to consider a significantly reduced data package to support the import of highly purified raw and refined sugar.

Finally, the general approach used in this study could be expanded to include other processed food ingredients derived from GM plants, including products like oils, oil fractions, lecithin and vitamins, where the processes are well established and the quantity of newly-expressed proteins are present consistently at *very low concentrations*.

AUTHOR CONTRIBUTIONS

JH and RL: conceived the study; JH and RK: analyzed the data; RK, JH, and AC-G: wrote the manuscript; AC-G and WdO: provided supportive information.

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Conflict of Interest Statement: The authors of this publication, RK, RL and JH are consultants to CTC on biosafety studies of products related to the research being reported. The authors of this publication, AC and WdO, are employees of CTC which is developing products related to the research being reported.

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OPINION
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Recovering the Original Intentions of Risk Assessment and Management of Genetically Modified Organisms in the European Union

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Keywords: GMO, EU, risk assessment, risk management, regulation

EARLY DRAFTING OF THE GMO REGULATIONS

Progress in the field of recombinant nucleic acid techniques and cross-species gene delivery in the 1970s and 1980s prompted legislators in the European Union (EU) to develop biosafety regulations encompassing these techniques and their resulting products. The ensuing procedure for risk assessment and risk management of genetically modified organisms (GMOs), as these were denominated, in the EU has thus been established with the purpose of ensuring a high level of protection of human health and the environment. The early draft legislative texts on GMOs in the EU (Commission of the European Communities, 1988) resulted in the first Council Directive on the deliberate release into the environment of GMOs (Dir 90/220/EEC) in 1990 (Official Journal of the European Communities, 1990). From these early drafts, it is clear that the intentions were to have an evolving and increasingly trait-oriented regulatory framework taking into account technical developments, potential safe history of use as well as potential benefits resulting from the application of these techniques and their resulting products. However, despite nearly three decades of research, product development, demonstrated benefits and a lack of demonstrated risks associated with recombinant nucleic acids *per se*, the GMO regulatory framework in the EU has neither evolved nor been implemented as intended. I here list four details for which policy makers in the EU need to consider the original intentions of the GMO regulatory framework in order to correctly interpret the current legislative texts as well as allow for necessary updates following technical progress.

Shifting Focus to Organisms and Their Traits

There is much unnecessary confusion nowadays on whether the EU is regulating GMOs on basis of the techniques that were applied or on the nature of the resulting organisms and their derived products. According to the EU Directive 2001/18/EC, a GMO is defined as *"an organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination"* (Official Journal of the European Communities, 2001). Custers (2017) points out that this definition is somewhat ambiguous regarding the interpretation of "altered in a way," and shows that Annex 1A, part 1, of the same Directive gives further indications by stating that *"Techniques of genetic modification referred to in Article 2(2)(a) are inter alia: (1) recombinant nucleic acid techniques involving the formation of new combinations of genetic material [. . .] and their incorporation into a host organism in which they do not naturally occur."* This means that within the EU regulatory framework a GMO is achieved only when the application of a particular technique leads to a particular result, i.e., an organism carrying artificially recombined nucleic acids in novel formation. This view is also shared by other authors (Sprink et al., 2016a,b; Kahrmann et al., 2017) as well as by the European Commission (European Parliament, 2014).

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If we go back to the early legislative drafts, it is clear that the intention has always been to regulate the resulting organisms and their derived products as much as, or perhaps to an even higher degree than, the underlying techniques. In the 1988 proposal for a Council Directive on the deliberate release to the environment of genetically modified organisms (GMOs), it was suggested that "*The present approach, which focusses on the new techniques of genetic engineering, is the first and most urgent step in the regulatory process; however, this will not impede evolution towards a more organism-related approach*". Along with this aspiration, it was also noted that "*different categories of organisms and/or techniques may be established, allowing different requirements for organisms of different levels of risks*" (Commission of the European Communities, 1988). Models for setting up different risk-based categories of organisms and/or techniques have already been developed (Barton et al., 1997; Miller, 2010; Beker et al., 2016; Conko et al., 2016; Ricroch et al., 2016), whereas the EU is currently in practice arguably very far away from living up to these original intentions. Zetterberg and Edvardsson Björnberg (2017) have also recently suggested that a new protocol for risk assessment incorporating selected aspects of traits and gene functions, rather than the mere presence of recombinant nucleic acids in the product, may contribute to making the EU GMO legislation more consistent regarding criteria such as non-discrimination of techniques and scientific adaptability taking the latest scientific findings into account. In this context, it is also worth noting that the Norwegian Biotechnology Advisory Board has initiated a public discussion on the future regulation of gene technologies, asking if there is a need for new dividing lines (Norwegian Biotechnology Advisory Board, 2018). Norway is not an EU member state but, being part of the European Economic Area (EEA), has implemented the EU Directive 2001/18/EC in its Gene Technology Act (GTA). A level-based approval system is now being suggested for the discussion, based on the type and extent of genetic change. Other criteria are also relevant, including altered traits, the intended use of the organism, the risk to health or the environment, sustainability, societal benefits and ethics.

Periodical Updating of Annexes

On 1st September 2017, the Netherlands published a proposal to improve the exemption mechanism for GM plants under Directive 2001/18/EC.[1] This proposal was put forward to resolve the decade-long issue of the regulatory status of new plant breeding techniques (NPBTs), and suggests to amend Annex 1B of Directive 2001/18/EC which lists GM techniques yielding organisms that are excluded from the Directive. The amendment would add a list of criteria, exempting from regulation plants that (1) do not contain other genetic material than from the same, or a crossable, species and (2) do not contain recombinant nucleic acids. Not in any way redefining the logic of Directive 2001/18/EC, the proposal from the Netherlands is a congruent way to clarify the GMO definition, including what is regulated and what is not, beyond any reasonable doubt and provide a practical solution to handle certain NPBTs.

It is also perfectly aligned with the original intentions for the GMO regulatory framework in the EU. The 1988 proposal for a Council Directive advertises "*the commitment to update the Directive to technical progress as necessary, given the rapid scientific development of this field*" and declares further that "*the Commission shall adapt the annexes of this Directive to technical progress by amending new techniques to be covered or deleting as appropriate*" (Commission of the European Communities, 1988). However, the provision to amend the Annex listing techniques that yield or do not yield GMOs was not included in later Directives (Official Journal of the European Communities, 1990, 2001). However, nearly 30 years of technical progress is arguably a compelling reason to endow the current Directive with such a mechanism and the proposal from the Netherlands also suggests that a review process for periodical adaptations to technological progress should be designed.

The idea has been up for discussion before. In 2006, a research team from Wageningen University and Research Centre in the Netherlands proposed to add cisgenesis to Annex 1B of Directive 2001/18/EC on the grounds that the resulting plants are similar to traditionally bred plants (Schouten et al., 2006a,b). Though being criticized for not approaching the issue of whether or not the phylogenetic distance of donor and recipient organism is relevant to risk assessment (Giddings, 2006; Eriksson et al., 2014), it nevertheless provides important clues to the regulatory status of NPBTs given the current GMO regulatory framework in the EU and the definition contained therein.

Acknowledging the History of Safe Use

Several GMOs and their derived products have been on the market in many countries and regions, including the EU, for more than two decades and these specific applications now arguably have a long safety record. Several reviews on GMO safety research demonstrates that no significant hazards directly associated with the use of recombinant nucleic acid techniques have been detected so far (Domingo and Giné Bordonaba, 2011; DeFrancesco, 2013; Nicolia et al., 2013). The International Centre for Genetic Engineering and Biotechnology (ICGEB) also provides a comprehensive collection of many thousands of scientific articles published since 1990 on biosafety and risk assessment in biotechnology.[2]

Directive 2001/18/EC states that its provisions should not apply to organisms which have conventionally been used and have a long safety record, however the same Directive lacks any indication of how to apply this very criterion of "long safety record" (Official Journal of the European Communities, 2001). The intention to take a long safety record into account was present already in the drafting of the first GMO legislation in the EU: "*The techniques not covered are those that have long been used with crop plants and livestock with an excellent safety record*". That recombinant nucleic acid techniques were relatively new and untested in the 1980s was also emphasized several times: "*In*

[1] https://www.rijksoverheid.nl/binaries/rijksoverheid/documenten/kamerstukken/2017/09/13/proposal-for-discussion/proposal-for-discussion.pdf

[2] http://bibliosafety.icgeb.org/

a largely unexplored field like this, the exchange of information is likely to play an essential role in gaining experience" (Commission of the European Communities, 1988).

Let us compare with conventional induced mutation breeding, which started to be applied large-scale in the 1950s (Oladosu et al., 2015). When the first GMO legislation in the EU was being developed in the late 1980s, induced mutations thus had a history of safe use stretching over more than 30 years and were therefore exempt from the regulatory provisions applied to GMOs. Today, GMOs have been used safely in commercial applications for more than 20 years and scrutinized in research and through market authorization requirements already significantly more than induced mutation breeding. One may therefore ponder the rhetorical question put by DeFrancesco (2013): "*How safe does transgenic food need to be?*". We now have plenty of evidence that recombinant nucleic acid techniques are not inherently unsafe and the responsible policy makers should therefore consider to modify the regulatory requirements accordingly, in part by shifting focus to organisms and their traits and also to initiate discussions on how to implement a model with risk categories based on traits and/or techniques as mentioned above.

Acknowledging Potential Benefits

Plenty of reviews and meta-analyses have demonstrated the environmental, agricultural and economic benefits of certain GMOs and their derived products (Qaim, 2009; Fagerström and Wibe, 2012; Green, 2012; Mannion and Morse, 2012; Klümper and Qaim, 2014; Brookes and Barfoot, 2017). The 1988 proposal for a Council Directive on GMOs predicted the potential for these benefits and added that "*It must also be acknowledged that the use of GMOs could lead to improvements in health and the environment by permitting the development of more precise agricultural inputs for protection and nutrition*" (Commission of the European Communities, 1988). However, this acknowledgement is absent from the later Directives (Official Journal of the European Communities, 1990; 2001). Part of the general provisions of the current Directive 2001/18/EC is though that "*In accordance with the precautionary principle, the objective of this Directive is to approximate the laws, regulations and administrative provisions of the Member States and to protect human health and the environment*" (Official Journal of the European Communities, 2001). In light of the overwhelming evidence of benefits demonstrated by the references listed above as well as the absence of associated risks, it can be argued that certain applications of GMOs and/or their derived products are compatible with an interpretation of the precautionary principle that would promote, rather than prohibit, these applications. Returning to the analysis by Zetterberg and Edvardsson Björnberg (2017), they also suggest an alternative regulatory model based on sustainability criteria that apply to all varieties regardless of the applied breeding methods. This model would certainly be compatible with the precautionary principle as the primary goal would not be to merely avoid risk by refraining from the use of certain techniques but instead to achieve a broader set of sustainability goals.

CONCLUSIONS

I have here argued that the regulatory framework for GMOs in the EU, and its implementation, has deviated considerably from the original intentions three decades ago when the fields of recombinant nucleic acid techniques and cross-species gene transfer were still relatively new in research and commercial applications. Given the experienced benefits of GMO applications, the safe history of use and the technical progress in the field of gene technologies, it is imperative to bring the GMO regulatory framework back in line with the original intentions and provide for a more trait- and benefit-oriented interpretation. The four above listed details provide starting points for discussions among policy makers in the EU.

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Management of Field-Evolved Resistance to Bt Maize in Argentina: A Multi-Institutional Approach

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Evolution of resistance to control measures in insect populations is a natural process, and management practices are intended to delay or mitigate resistance when it occurs. During the 2012/13 season the first reports of unexpected damage by *Diatraea saccharalis* on some Bt maize hybrids occurred in the northeast of San Luis province, Argentina. The affected Bt technologies were Herculex I® (HX-TC1507) and VT3PRO® (MON 89034 × MON 88017*). Event TC1507 expresses Cry1F and event MON 89034 expresses Cry1A.105 and Cry2Ab2, whichr are all Bt proteins with activity against the lepidopterans *D. saccharalis* and *Spodoptera frugiperda* (MON 88017 expresses the protein Cry3Bb1 for control of coleopteran insects and the enzyme CP4EPSPS for glyphosate tolerance). The affected area is an isolated region surrounded by sierra systems to the northeast and west, with a hot semi-arid climate, long frost-free period, warm winters, hot dry summers, and woody shrubs as native flora. To manage and mitigate the development of resistance, joint actions were taken by the industry, growers and Governmental Agencies. Hybrids expressing Vip3A protein (event MIR162) and/or Cry1Ab protein (events MON 810 and Bt11) as single or stacked events are used in early plantings to control the first generations of *D. saccharalis*, and in later plantings date's technologies with good control of *S. frugiperda*. A commitment was made to plant the refuge, and pest damage is monitored. As a result, maize production in the area is sustainable and profitable with yields above the average.

Keywords: Bt-maize, field evolved resistance, *Diatraea saccharalis*, sugarcane corn borer, Argentina, mitigation plan, insect resistance management (IRM)

INTRODUCTION

Insect pests are one of the main causes of losses in agriculture. Many different tools are available for farmers to manage insect pests. Since 1998 in Argentina maize farmers have grown transgenic hybrids that express insecticidal proteins derived from *Bacillus thuringiensis* (Bt maize) as an effective and environmentally friendly tool for integrated pest management

(IPM). Evolution of resistance to control measures in insect populations is a natural process, and insect resistance management (IRM) practices are intended to delay or mitigate resistance when it occurs. One of the key measures for delaying evolution of resistance is the implementation of a refuge area in a Bt plot. The refuge is a portion of the field planted with non-Bt seeds where susceptible insects can survive to preserve susceptible alleles in the population. In the case of Bt maize in Argentina, the recommended refuge proportion is 10%. During the 2012/13 season the first case of field evolved resistance to Bt maize occurred in Argentina. *Diatraea saccharalis* (sugarcane borer, SCB) produced greater than expected damage on two Bt technologies: Herculex I® (Hx-TC1507) and VT3PRO® (MON 89034 × MON 88017) (Signorini et al., 2017; Grimi et al., 2018), both events express Bt proteins with activity against SCB and *Spodoptera frugiperda* (fall armyworm, FAW). The area affected is an isolated region and was not part of the major maize producing area until 2005 when farmers invested in pivot irrigation together with a high rate of Bt technology adoption.

When the unexpected damage was detected in 2013 farmers contacted technology providers to understand the situation and obtain recommendations on mitigating yield loss. The companies (Dow AgroSciences, DuPont Pioneer and Monsanto) worked together through the Argentina Seed Associations with the farmers and with the multiple governmental agencies involved in the approval and commercialization of transgenic crops (also known as genetically modified organisms GMOs) in Argentina. This article describes the joint actions taken by the industry, growers and Governmental Agencies to manage and mitigate this first case of resistance to a Bt crop in Argentina and the resulting improvement in management of SCB.

DESCRIPTION OF THE PROBLEM SITUATION

Environment and Agro-Ecologic Description

The department of Ayacucho in the northeast of San Luis province, where the greatest damage was detected, is geographically isolated by mountains from other maize producing areas. It has a dry environment, low rainfall with high temperatures in summer and mild winters with long frost-free periods and natural shrubby vegetation. Crop management under these environmental conditions is atypical of maize production areas in Argentina. Maize production requires irrigation and planting occurs during an extended period from September to January, with double cropping possible. These practices result in maize crops that are available almost year-round and more attractive to insects than other vegetation in the region. The agro-ecological characteristics of the area and the installation of pivot irrigation made this area a good place for production of maize seed making this a profitable activity that occupied between 30 and 50% of the area planted with maize.

Bt Adoption and Technology Management

These environmental conditions and agricultural practices led to intense insect pest pressure in northeastern San Luis. This high insect pressure limited the planting date of maize, only to September and beginning of October, timing that has a high demand for irrigation. Upon commercialization, Bt maize provided a new tool for growers to manage lepidopteran pests that was more effective and simpler to deploy than insecticidal sprays and cultural practices. Accordingly, adoption of Bt technologies in the area was very rapid, especially for late-season plantings in which FAW is the major pest. By allowing late planting dates, a greater efficiency in the use of irrigation water was achieved, and yields were stabilized. Herculex I was launched in 2005 and provided very good control of SCB and FAW and farmers adopted this technology extensively across all the planting dates. In 2010 VT3PRO was commercially approved and adoption was again rapid (**Figure 1**).

In most cases, IRM practices (e.g., refuge adoption, crop rotation, weed management, insect monitoring, and insecticide applications when pest populations reached economic thresholds) were not emphasized with growers. These factors are similar to other resistance cases reported in the world and may have contributed to an unusually high selection pressure for SCB populations to evolve resistance northeastern San Luis.

Damage

Upon reports of unexpected damage in HX or VT3PRO hybrids by growers, the technology providers visited the affected area to identify the damage, identify the pest species involved, and ensure that seed quality was not an issue. Once the pest was identified as SCB and the hybrids were confirmed to be HX or VT3Pro several fields were scouted to understand the extent (number of hectares) and the severity of the damage. Results showed that the affected area included nearly 11,000 hectares, including 9,000 ha with pivot irrigation in intensive agriculture and 2,000 ha of lower technology and land irrigation.

The damage found consisted of severely bored stems, tunnels that were over one meter long, and often more than one gallery per stem. This caused plant stem breakage, poor grain filling (low grain quality), and reduced yield (**Figure 2**). Sugarcane borer damage in stubble from the previous year indicated that the situation had existed for at least one season.

Insects surviving on maize containing these events are called "resistant biotype" after confirming the resistant phenotype (Signorini et al., 2017; Grimi et al., 2018) since no populations genetics studies have been done to determine whether all the insects in the whole geographic area consists of a single genetic population.

PARTIES INVOLVED

As with any case of reduced benefits of a technology, many parties are affected and involved in different ways. In the case of field-evolved resistance of a target pest to a Bt crop, the main parties affected are farmers with resistant populations in their fields, technology developers, and, in Argentina, governmental agencies that regulate different aspects of the agricultural productions. For

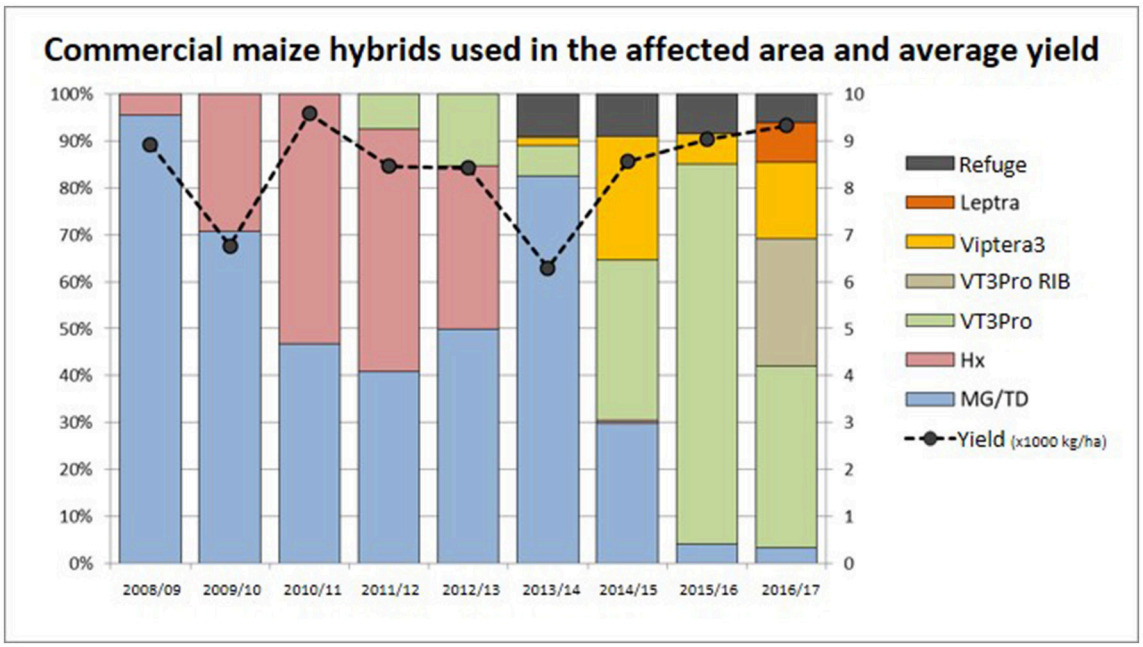

FIGURE 1 | Technologies planted and average yield for maize in each season in the affected area. The bars represent the percentage of each technology planted in the affected area, and the dotted line indicates the average yield obtained for maize in the same area in thousand kilograms per hectare (Source: A. Cadile, CREA Brochero). [Leptra is the marketing name for the breeding stack of maize events TC1507 × MON810 × MIR162 × NK603. Viptera3 here refers to the breeding stack of maize events Bt11 × GA21 × MIR162. MG/TD here refers to Cry1Ab-Bt maize corresponding to events MON810 (MG) or Bt11 (TD)].

field-evolved resistance in a main target pest to Bt maize, three governmental institutions are related with different involvement: the Directorate of Biotechnology, the National Seed Institute, and the Directorate of Surveillance and Monitoring.

The Directorate of Biotechnology (*Dirección de Biotecnología*, DB)[1] (within the scope of the Secretary of Food and Bioeconomy within the Ministry of Agroindustry) is in charge of the environmental risk assessment for the release of a GMO into the agricultural environment upon consultation with the National Advisory Commission on Agricultural Biotechnology (CONABIA). The CONABIA is constituted by representatives of the public and private sector involved in agricultural biotechnology. It is an interdisciplinary and inter-institutional group. It is recognized that resistance of target insects to Bt crops is not a biosafety risk but rather causes a reduction in the value or benefit of the Bt crop. Accordingly, the environmental assessment of an event for commercialization includes a section on IRM as an institutional way of helping preserve the benefits that the Bt technologies have for the environment and agricultural production in Argentina (Resolución SAGyP N° 701/11)[2].

FIGURE 2 | Damage caused by *Diatraea saccharalis*.

The National Seed Institute (*Instituto Nacional de Semillas*, INASE) oversees the proper execution of the Seed Law in Argentina. Among other objectives, the INASE issues resolutions related to seed production, hybrid registration, certification of seeds from origin, and packaging and labeling of the final product (seed bags). INASE is a member institution of CONABIA, thus it is involved also with GMO regulations.

The Directorate of Surveillance and Monitoring (*Dirección de Vigilancia y Monitoreo*, DVyM) within the National Service for Agri-food Health and Quality (*Servicio Nacional de Sanidad y Calidad Agroalimentaria*, SENASA) is in charge of the Argentine National System of Pest Monitoring and Surveillance

[1]Dirección de Biotecnología (2014) Circular N°7. Directrices para la evaluación de los ítems D13 y D14 del formulario de segunda fase.

[2]Ministerio de Agroindustria (2011) *Resolución SAGyP N° 701/11* Available online at: https://www.agroindustria.gob.ar/sitio/areas/biotecnologia/solicitudes/___experimental/_archivos/resolucion%20OVGM%20701-2011.pdf.

(SINAVIMO), whose general goal is to provide updated and trustful information on the status of plant and animal pests, including those with impact on the productivity of the crops and cattle. Additionally, at the time of the sugarcane borer resistance case (2012/13), this Directorate received also the technical support of the National Commission of Resistant Pests (*Comisión Nacional de Plagas Resistentes*, CONAPRE).

The Argentine Seed Association (ASA) is a chamber that joins more than 80 seed companies, developers and licensees of agrobiotechnology, in the country. ASA coordinates member discussions on common themes that affect seed industry, like regulatory affairs, intellectual property and stewardship topics, among others. It has a technical and communication working group on IRM that is integrated by member companies with the main goal of preserving Bt technologies.

Most of the growers in the affected area, members of CREA Brochero, were grouped in an "Irrigation Group" that had regular meetings where they exchange experiences and knowledge. The existence of this group facilitated the alignment between growers and the work with the industry and governmental agencies.

APPROACHING THE PROBLEM TOGETHER

Since many of the stakeholders described above have common interests in the general scope of agriculture and productivity for Argentina, there is a long history and experience of all parties working together. Field evolved resistance of sugarcane borer to Bt maize in San Luis was a new situation for which a solution had to be reached with a multi-institutional partnership.

After the detection and confirmation of unexpected damage to Bt maize by SCB, several meetings between growers, industry and governmental agencies took place over the following months. The situation was communicated to the Maize Chain Association and Growers Associations in August 2013, and academic experts were consulted to gather as many perspectives as possible for this first case in Argentina. The existing relationship between ASA and governmental agencies, which started in 1998 when the first Bt maize was commercialized in Argentina, was key for this multi-stakeholder collaboration to be effective.

Initially, the technology providers and growers were trying to assign blame to one another, and were reluctant to identify their own mistakes. Over the course of these meetings, a common understanding emerged that the situation had to be approached together by all the parties involved as agricultural community with a common goal: sustain maize productivity in the affected area (as maize is a key component in the system) and as far as possible keep the resistance confined to the original location.

A mitigation plan was discussed based on the previous characterization, and on the risk of spread of the resistant biotype. Several measures were proposed, some of them very radical and difficult to implement except in specific cases (not planting maize, tillage, not planting wheat or other SCB hosts, etc.). Other proposed measures were easier to implement. A plan for monitoring pest damage was developed with thresholds set for the application of chemical insecticides. For this purpose, a service company was hired to monitor around 9,000 ha included in the plan. The monitoring included, at least, a weekly visit to each field to detect egg masses, and upon detection of 10% plants with colored egg postures (indicating less than a week to hatching) persistent products were recommended for chemical control according to well established threshold by referent entomologists (Iannone and Leiva, 2015). A commitment was made to plant the refuge, and it was decided that Bt materials expressing different modes of action (e.g., Cry1Ab or Vip3A protein as in event MIR162) would be used exclusively (as they had not been affected by the resistant biotype). During the first season of the plan (season 2013/14), there was high FAW pressure, which caused severe damage to Cry1Ab maize, even in early plantings (since Cry1Ab maize does not provide protection from high FAW populations). Maize productivity of that season was the lowest in the last 10 years. Due to this situation, the following season it was agreed to use Cry1Ab or Vip3A technologies (single or stacks) in early planting to control the first generations of SCB, and in late planting it was necessary to use events with good control against FAW. Thus, technologies containing TC1507 or MON89034 events, either as single or stacks (such as Leptra or VT3Pro), had to be reintroduced in the affected area and new events as Viptera3 (containing Vip3A and Cry1Ab Bt proteins) were introduced to control this relevant pest (**Figure 1**).

This Mitigation Plan was communicated by ASA to the three governmental agencies. The DB together with CONABIA collaborated by providing technical assistance coming from their experts, and by helping delineate new regulations for IRM for Bt crops. The development of the Guideline (Circular N°7/2014) was coordinated by the DB and the actors involved in carrying this task were CONABIA, the National Institute of Agricultural Technology (INTA), SENASA, INASE and ASA (representing the Bt seed providers from the private sector). This multiparty collaboration was essential to determine the information necessary for Bt developers to provide.

The DVyM also took an active role as the Institution with expertise in the field of pest resistance. Their previous experience in cases of weed resistance to herbicides (especially glyphosate-resistant *Sorghum halepense* in Argentina) was of great guidance for the development of the Mitigation Plan and the coordination of the multi-institutional work.

INASE took a proactive role in the confinement of the resistant biotype to the affected area. As the Agency responsible for the certification of seeds from origin, it issued a Resolution to prevent seed production in the affected area with the aim of avoiding the escape of larvae inside the cobs toward the regions where the seeds are conditioned and bagged (mostly in the maize belt of Argentina). The first Resolution (N° 328/2013)[3] was issued as early as September 2013, and annually renewed until its latest update in 2016 that made the measure permanent until new information becomes available that could justify the release of the restriction. This is a key measure from the containment perspective, but it produced many complaints by farmers that used to plant maize for seed production in the area (30–50% of maize area before the resistance outbreak).

[3]INASE (2013). *Resolución N° 328/2013*: Available online at: http://servicios.infoleg.gob.ar/infolegInternet/anexos/215000-219999/219950/norma.htm.

Additionally, INASE involved its Summer Crops Commission (CONASE) for the understanding and support of additional IRM measures, leading to a Resolution to enable the seed blend refuge strategy in Argentina (Resolution N° 112/2014)[4]. The seed blend refuge, also known as refuge in the bag (RIB), is an alternative strategy of refuge where the Bt and non-Bt seeds are already mixed in the bag so that when farmers plant the bag they are planting Bt and its refuge at the same time. For the current Bt maize commercialized in RIB format in Argentina, the blend refuge is a mixture of 10% non-Bt seeds and 90% Bt seeds. This refuge strategy makes the operative part easier for the farmer while it guarantees the refuge will be more effective since it is planted at the same time as the Bt plants, with hybrids of similar maturity and agronomic practices and in the right proportion. So far, refuge seed blends are recommended for the areas in Argentina where SCB is the main pest since interplant movement by larvae is limited.

CURRENT SITUATION

Despite the presence of sugarcane borer resistant biotype, at present damage caused by sugarcane borer and fall armyworm is limited and maize production in the area is sustainable and profitable with yields above the average. IPM and refuge education and training are continuing, together with INASE's prohibition of maize seed production in the affected area. Currently maize can be planted from the beginning of September to January and the Bt technology used is selected considering the planting date, the position in crop rotation, and neighbor crops. Damage by both SCB and FAW, is limited. The refuge adoption is high: 75% of the area planted with Bt maize included the corresponding refuge in 2013/14 season, increasing to 87% of compliance in 2014/15 season. **Figure 1** reflects this successful compliance showing that 10% of the volume planted with maize corresponded to refuge in season 2013/14 to 2015/16 and the addition of RIB for VT3Pro in season 2016/17 (data provided by CREA Brochero, **Figure 1**). Except for some refuge management, it is practically not necessary to make insecticide applications to manage these pests, when the technology is well positioned in the system.

[4]INASE (2014). *Resolución 112/14:* Available online at: http://servicios.infoleg.gob.ar/infolegInternet/verNorma.do?id=230560.

CONCLUSIONS

This case generated many learnings that need to be emphasized. From the agronomical standpoint, the first learning showed that all parties involved had been working on the simplification of the agronomic practices, while the system is indeed complex. With this simplification of the system, the selection of resistance was accelerated, leading to a rapid loss of benefits. From a social perspective, another learning is that in order to preserve new technologies education is a key factor, and for Bt technologies this includes training farmers on Best Management Practices and improving refuge compliance. Preservation of the technology can only be reached through collaboration among all parties involved.

Resistance management and mitigation preserves the benefits of Bt crop technologies. This experience shows that with appropriate management practices maize can still be produced sustainably in an area where resistance to Bt events has occurred. The mitigation plan implemented by farmers, industry and government has been successful in limiting the spread of the resistant biotype. This positive scenario can only be reached because all parties involved have joined efforts toward this common goal.

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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Off-Patent Transgenic Events: Challenges and Opportunities for New Actors and Markets in Agriculture

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More than 20 years ago, the first genetically modified (GM) plants entered the seed market. The patents covering the first GM plants have begun to expire and these can now be considered as Off-Patent Events. Here we describe the challenges that will be faced by a Secondary Party by further use and development of these Off-Patent Events. Indeed, the conditions for Off-Patent Events are not available yet to form the basis for a new viable industry similar to the generic manufacturers of agrochemicals or pharmaceutical products, primarily because of (i) unharmonized global regulatory requirements for GM organisms, (ii) inaccessibility of regulatory submissions and data, and (iii) potential difficulties to obtain seeds and genetic material of the unique genotypes used to generate regulatory data. We propose certain adaptations by comparing what has been done in the agrochemical and pharmaceutical markets to facilitate the development of generics. Finally, we present opportunities that still exist for further development of Off-Patent Events in collaboration with Proprietary Regulatory Property Holders in emerging markets, provided (i) various countries approve these events without additional regulatory burdens (i.e., acceptance of the concept of data transportability), and (ii) local breeders agree to meet product stewardship requirements.

Keywords: off-patent event, generic, transgenic, GMO, data transportability, emerging markets

INTRODUCTION

The first genetically modified (GM) plants were produced early in the 1980s by means of *Agrobacterium tumefaciens* as a vector to introduce a new gene into the plant as a trait of interest (Bevan et al., 1983; Herrera-Estrella et al., 1983). Numerous laboratories from the public and private sectors have worked on the production of GM plants, leading to the first commercial GM plants in the mid-1990s (James and Krattiger, 1996). Since then, many new GM crops have reached the market and been adopted all over the world. In 2016, more than 18 million farmers grew GM crops on a total of 185.1 million hectares in 26 countries, a 110-fold increase since the first releases (James, 2016), demonstrating the very successful adoption in global cropping systems despite intense societal debates. The main traits commercialized are herbicide tolerance and/or insect resistance

(James, 2016). More than 20 years after the initial commercialization, patents covering these profit-making Events have begun to expire. These patents were valid for 20 years after granting in the USA and Canada and after filing in other countries. In contrast to generic product development in the pharmaceutical and agrochemical industries, the current regulatory regimes for GM crops make it particularly challenging and currently virtually impossible to establish a viable generic industry in this sector.

The timeline for commercialization of an Event is long (∼14 years for the first commercial launch) (Fraley, 2015) and the investment high (McDougall, 2011), in particular to comply with all the regulatory requirements, address the stewardship expectations, and assume the liabilities associated with GM crops, thereby reducing the market opportunities to only a few companies for limited crop/trait combinations.

Proprietary Regulatory Property (PRP) Holders must maintain regulatory approvals in the countries in which they intend to release Events for cultivation as well as in countries where plants containing the Event or the GM plant-derived products will be exported. In many countries, such approvals are limited in time and need to be renewed regularly. PRP Holders must also observe stewardship requirements and remain legally responsible for all issues related to product identity, quality, and performance. When the Event becomes off-patent, these requirements remain in force if the PRP Holder wishes to maintain the sales or if a Secondary Party wishes to commercialize the Event. Jefferson et al. (2015) provided insights into some of the challenges to be addressed in post-patent use of GM crops.

Here we discuss the difficulties faced by any potential Secondary Party who wishes to use or further develop these Off-Patent Events, among which are (i) lack of harmonization of the global regulatory requirements for GM crops, (ii) limited accessibility to regulatory submissions and data, and (iii) potential obstacles to obtain material of the unique Event upon which the regulatory dossier was created. Notwithstanding this problematic context, we present existing opportunities to further develop Off-Patent Event plants in collaboration with PRP Holders in new markets, provided that the concept of data transportability becomes widely accepted and that the product stewardship and the regulatory requirements are observed by all users at the global level.

We will not cover the generation of a Generic Event (see Glossary) that differs from an Off-Patent Event (see Glossary). The development of a Generic Event requires a complete regulatory package, even when some data on specific components of the Event can be obtained from the PRP Holder or are publicly available. Recently, an approach has been reported to produce generic glyphosate-tolerant soybean (*Glycine max*) (Rojas Arias et al., 2017). Regulatory and stewardship responsibilities for a Generic Event will be the same as for an Off-Patent Event and this will also be true for the liabilities that could be even more challenging for the developer of a Generic Event, which will probably be unpatentable.

OFF-PATENT EVENTS ARE NOT GENERIC

Generic products are widespread in the pharmaceutical or agrochemical industries because of the specific legislation that facilitates their commercialization. In these agrochemical or pharmaceutical products, the off-patent active ingredient is a molecule or a protein ("biosimilar") and is the same (or "similar" for a protein) as in the original product, even when the production process is different. In addition, the formulation of the active ingredient can have been modified (Alfonso-Cristancho et al., 2015). Although specific procedures were developed to facilitate the registration of generic or biosimilar products (such as possibilities for data bridging in regulatory applications and authorization to initiate a regulatory package of a product before expiration of the corresponding patent), generic products have to obtain their own commercial authorizations.

In the case of GM plants, the situation differs, because the intellectual property coverage does not protect a molecule or a protein, but an Event, and no legislation has yet been put in place in any country to facilitate the conditions for development, sale, and use of Off-Patent Events. Below, we will focus on the challenges to be faced by any Secondary Party wishing to further develop and use an Off-Patent Event.

INTELLECTUAL PROPERTY RIGHTS

An Event can be protected by several patents, covering, for instance, the DNA sequences used (promoter, coding sequence,…) to obtain the new trait, the technologies used to produce the Event, the Event itself, its use, and the specific detection tests used to identify its presence. Ten patents cover the soybean Event GTS-40-3-2 in the USA (Jefferson et al., 2015). When ascertaining that an Event is off-patent, one has to check the expiration of all the patents in the considered countries, i.e., countries for cultivation and for import, that cover the Event itself, its use, and its derived products. Indeed, one should take into account that the commercialized Event may not be patented as such, but any plant containing the construct for the trait and that, hence, the patent concerning the plant would also cover the commercial Event.

Even when an Event is off-patent, the commercial varieties derived from this Event may still be protected, either through a patent in the USA or through the Plant Variety Protection (PVP) Act in most other countries. In the USA, patented varieties cannot be used for breeding, whereas in Europe, for example, it is allowed to breed varieties under PVP. In this latter case, the derived varieties can be freely commercialized if the patented trait has been removed. However, if the trait is still patented, a license from the patent trait owner is necessary for as long as that patent is in force.

Let's assume that the Event and its derived varieties are completely off-patent, then the PRP Holder would be confronted by the situation in which unlicensed Secondary Parties could use the Off-Patent Event for breeding (for instance, to develop new varieties) and cultivation (for instance via farm-saved seeds). An unlicensed Secondary Party could possibly also utilize the Off-Patent Event to generate varieties with combined traits

(i.e., "stacked" Events), but, in that case, the Secondary Party would require the necessary technical ability and possess the PRP-related information to fulfill the regulatory conditions for such stacked Events. Potential candidates would include seed companies experienced in developing and managing Events, public institutions capable of creating their own varieties under license from the PRP Holder (such as the University of Arkansas) (Miller, 2016), and individual farmers able to grow farm-saved seeds, provided again no previously signed technology use agreement exists with the PRP Holder that prohibits saving seeds for subsequent cultivation and that local legislation and licenses allow its application to purchased seed bags. In contrast, licensed seed companies are required to use the Event only in accordance with the terms of their license agreement that usually contain restrictions on its use, independently of the patent coverage, and generally, such restrictions survive the termination or expiration of the license agreement. In other words, an Off-Patent Event cannot be used in a manner that is not permitted by the license terms. The same terms would apply also to farmers who have signed a technology use agreement with the PRP Holder.

Thus, as the intellectual property rights of the Event and its derived varieties expire, the PRP Holder has to reconsider the value capture mechanisms and decide in due time on possible options: (i) continue the commercialization on its own and/or reach an agreement with Secondary Parties interested in the use of the Off-Patent Event, or (ii) discontinue sales and regulatory approvals. In this decision process, the market opportunity will be considered for stacked Events, in which the Off-Patent Event is combined via breeding with other Events, possibly still protected by intellectual property rights. Such combinations may allow novel applications of the Off-Patent Event.

Should a Secondary Party wish to develop, market, or use an Off-Patent Event independently from the PRP Holder, aspects related to the material, the regulatory requirements, and the stewardship should be taken into account.

REPRESENTATIVE PLANT MATERIAL OF THE OFF-PATENT EVENT

A Secondary Party interested in the use of an Off-Patent Event must first obtain legal access to the Event. If the Event itself has been patented, then seeds have generally been deposited in an International Depository Authority (IDA) under the Budapest treaty (WIPO, 2018), such as the American Type Culture Collection (ATCC) in the USA or the National Collections of Industrial, Food and Marine Bacteria (NCIMB) in the UK. After patent issuance, such deposited biological material must be made freely available to the public. The storage time in an IDA is at least 30 years (WIPO, 2002). However, a sample requested during the patent validity may not be used by the purchaser for any commercial use, because it would constitute a patent infringement. Moreover, under the ATCC Material Transfer Agreement, ATCC Material and Progeny "may only be used by the Purchaser's Investigator for research purposes and only in the Investigator's laboratory"—"Any commercial use of the Biological Material is strictly prohibited without the ATCC prior written consent" (Davis, 2011). Notwithstanding and independently of the ATCC restriction, under paragraph 5 of the Generic Event Marketability and Access Agreement (GEMAA), as amended on November 5 2015, PRP Holders agree to make the Event available to the GEMAA signatories (GEMAA, 2015).

REGULATORY STRATEGY

At the time an Event becomes Off-Patent in the major agricultural markets, the PRP Holder will have developed a global data package and obtained approvals for commercialization in countries in which the GM crop is intended for cultivation and for export in countries in which the harvested Event-containing plants, parts or GM plant-derived products will be imported. In the case of the United States Department of Agriculture-Animal and Plant Health Inspection Service (USDA-APHIS), once an Event is deregulated, it is considered equivalent to any other free article without need for follow-up submissions, unless data emerge that significantly change the risk assessment. In contrast to the USDA, in many other countries such as China, the EU, and South Korea, approvals are limited in time and resubmissions must be scheduled to renew approvals and avoid costly disruptions of international commodity trade. A resubmission may be a formal request for extension, but most authorities require additional information accounting for the acquired knowledge and even updates of previous studies to meet redefined needs since the original approval. A third type of approval (such as the procedures of the United States Environmental Protection Agency [US EPA] for Plant Incorporated Protectants) is even more restrictive: similar to chemical crop protection products, it is conditional, i.e., an approval will have mandatory performance and reporting obligations, such as implementation of an insect resistance management plan, and is granted directly to one particular party. In addition, such an EPA approval may be provisional. The distinction between the different types of approvals is important when the consequences of the off-patent situation are evaluated.

Although the PRP Holder will probably not stop supporting the regulatory approvals for the Off-Patent Events abruptly, no continuation will be guaranteed, especially if the PRP Holder intends to replace the Off-Patent Event with an improved patented Event. Thus, to be able to develop, breed, or use the Off-Patent Event, any Secondary Party must ensure that the necessary permits are and remain in place.

For a USDA-APHIS deregulation, there is no need to request a second deregulation. For time-limited approvals, the Secondary Party should monitor whether the approvals have been, or are in the process of being, renewed by the Primary PRP Holder. When approvals expire in a given country, the Secondary Party will have to cease any unapproved use in that country or obtain new approvals. Alternatively, the Secondary Party could apply for its own authorizations, possibly the only option in administrative systems that provide party-dependent authorizations (such as the US EPA), but associated with high regulatory costs due to compilation, submission, and maintenance of the authorization

and resulting in obligations for and liability of the Secondary Party.

In contrast to usually publicly available approvals, the PRP submissions are subject to confidentiality claims and are protected internationally under Article 21 of the Cartagena Protocol on Biosafety (Secretariat of the Convention on Biological Diversity, 2000) as well as possibly covered by copyright claims. The fact that some information is publicly available does not imply that it can be used to support a Second Party's own regulatory package or own application. For instance, in Europe, the regulatory system provides data protection to applicants, because Regulation 1829/2003/EU (Article 31) foresees that parties cannot use or refer to data submitted by the initial applicant in their application for 10 years, and under Directive 2001/18/EC (Article 25) the prohibition is unlimited in time.

In view of the difficulties for Secondary Parties to renew a particular approval (e.g., in China, South Korea, and the EU), it is very unlikely that Secondary Parties will have access to up-to-date information, because the data protection period starts from the submission date of specific information; in other words, Secondary Parties will not be allowed to use new information submitted as part of a resubmission until expiration of the protection period relevant for the renewal. However, if it is practically impossible and too expensive to establish its own complete safety package, a Secondary Party has always the possibility to negotiate access to submitted information with the PRP Holder.

SAFETY DATA PACKAGE

The GM Event safety is supported by a data package comprising studies explicitly providing information required by the decision makers. Whereas some information may be general, relevant to the trait (such as herbicide tolerance or insect resistance) or the gene (such as origin and nature of the nucleotide sequence and the corresponding protein) and be valid for several Events; most data, such as, for instance, the nucleotide sequence at the insertion site, the effect of the insertion on agronomical parameters, or the biological composition, are specific for each Event. In this case, it is important to demonstrate that the stud(y)(ies) has(ve) been conducted on the specific Event and molecular data and/or information on the genealogy of the material in support of the claims may be required by the authorities. The PRP Holder usually owns the study protocols and reports, and even when submitted as part of a data package, some level of protection may prevail or certain information may remain inaccessible due to confidentiality or copyright claims.

Whereas an initial data package serves to support market introduction, during the commercial lifetime of a GM crop additional data is accumulated and the data package is expanded. First of all, because the data package is submitted in various countries, the locally competent authorities may need specific data, requiring repetition of the initial study with an adapted study design or a completely new study. The PRP Holder can usually anticipate most requests for an acceptable study report but the problems expand when a country requires studies performed *in situ*. Secondly, over time, requirements change and are redefined, creating difficulties when a time-limited authorization expires and the authorities demand the submission of an up-to-date study design as part of the renewal. Finally, during large-scale implementation, unexpected findings might emerge that necessitate a specific effort to understand the discovery source and the impact on the risk assessment. In conclusion, the safety data package has to be substantially and continuously maintained throughout the life cycle of the Event, independently of its patent life. When a Secondary Party wants to independently engage in the use of an Off-Patent Event, a safety data package must be established as follows:

- by referring to the publicly available studies or data previously submitted by the PRP Holder, but usually not encompassing the entire safety package and,thus, rarely sufficient; indeed, the PRP Holder may have recently obtained information that has not been supplied to the competent authorities yet and, hence, are unknown and cannot be used by the Secondary Party;
- by agreeing with the PRP Holder on conditions for access to and use of the existing data;
- by establishing a proprietary data package by repeating the patented studies, requiring access to the biological material contained in the Off-Patent Event and legal permission to use such material for regulatory purposes. Under the current laws, at least in the USA, this data package can be initiated only when the Event or its constituents are off-patent.

COMPLIANCE WITH APPROVAL CONDITIONS

An additional regulatory aspect relates to the conditions and liabilities associated with the approval. Depending on the type of Event and its approval, specific stipulations may be imposed. The PRP Holder is responsible for ensuring that all specifications are implemented, possibly by transferring part of its obligations to licensees, including farmers, via contracts and technology use agreements. For example, specific labeling of the (Event-containing) GM products may be mandatory to inform the farmers about the nature of the material or about particular management practices. In some cases, the implementation of an insect resistance management plan is a prerequisite for the approval. These examples illustrate that the regulatory obligations of the PRP Holder do not stop at the approval, but need to be maintained rigorously during the lifetime of the Event.

Upon patent expiry, the leverage of the PRP Holder over other users is in principle reduced. Facing continuous and onerous regulatory obligations, but less well equipped to impose conditions, the PRP Holder will re-evaluate whether to comply with the regulatory requirements. In addition, when Secondary Parties will supply the same material as a new source, there is a risk that they may not comply with all the regulations imposed on the PRP Holder. More importantly, in the case of non-compliance or any unexpected finding, the PRP Holder will be the first to be questioned and from whom liability and redress will be sought. Therefore, the incentives for a PRP Holder to

discontinue sales and regulatory support for an Off-Patent Event and provide a new, patented Event as a substitute are extremely high.

PRODUCT STEWARDSHIP

Product Stewardship is the responsible management of a product from its launching through its use to its ultimate discontinuation. Although safety and compliance with legal obligations are inherent conditions to be observed, stewardship covers additional aspects of identity, purity, quality, and performance of GM crops and imposes a quality management system covering all Event handling that is subject to external audits. Furthermore, PRP Holders are expected to ensure the use of their products in a manner that respects the supply chain and does not disrupt international trade. The "Excellence Through Stewardship" (ETS) initiative was established by the biotechnology industry on a voluntary basis and promotes the adoption of stewardship programs and quality management systems across the full biotechnology plant product life cycle (www.excellencethroughstewardship.org). From this comprehensive stewardship program, some elements are particularly relevant for the discussion on Off-Patent Events. To avoid trade disruption, the developers (PRP holders) must ascertain that all required regulatory permits and authorizations are available in countries in which they intend to commercialize the Off-Patent Event and any derived products. For seed production, special care is taken to ensure the traceability and to avoid intermingling between non-GM and GM seed, as well as between different GM Events, requiring detailed knowledge of Event performance and characteristics, such as identity, genetic purity, and performance criteria. Along the value chain of the product, downstream users, i.e., farmers and downstream processing, need to be informed and trained for the optimal utilization of the Event, e.g., agricultural practices, labeling, channeling, and identity preservation. A specific case is the Integrated Pest Management (IPM) that aims at minimizing damage of pests, such as weeds, insects, and viruses, and maximizing the availability and longevity of the tools needed for the pest management. Irrespective of the impositions by the authorities, stewardship requires the developers to create their own IPM approach during the Research & Development phase, such as design of refuges of non-GM crops amidst GM insect-resistant crops. Due to the complexity of the process and the potential impact on multiple stakeholders, establishment of an Incident Response System is an essential part of any quality management system and is put in place as early as possible. Examples of incidents include improper functioning of the trait, an unintended, unauthorized release of the plant material in the environment, or a seed quality failure. Finally, developers must anticipate product discontinuation, as, for instance, when the commercial interest in a trait or particular Event has diminished and does not justify regulatory support continuation, implying a managing process to remove the specific Event from the market.

Any Secondary Party willing to further develop an Off-Patent Event will have to establish a stewardship program for the different development and commercialization steps of the Off-Patent Event. For practical purposes, in the USA, in view of the conditions imposed in article 13(a) of the GEMAA (GEMAA, 2015), the Secondary Party will have to become an ETS member and accept to be regularly externally audited. In addition, when such developments are done in collaboration with the PRP Holder, this PRP Holder may oblige the Secondary Party to have an audit system comparable to ETS.

THE PRECEDENT OF THE AGACCORD

To date, only the USA (through a voluntary, industry-negotiated agreement) established a framework agreement to manage Off-Patent Events, designated the AgAccord (www.agaccord.org). This framework comprises two separate agreements that cover the full spectrum of issues related to patent expiration: the GEMAA^{SM} (GEMAA, 2015) and the Data Use and Compensation Agreement (DUCA). The AgAccord supports business opportunities for parties seeking to use Off-Patent Events in the USA, while ensuring that all global regulatory commitments are maintained for Off-Patent Events and that the USA exports of the event-containing products are not disrupted. The AgAccord establishes a standard process to make available Off-Patent Events and the corresponding proprietary regulatory information otherwise not accessible to interested parties. In addition, this access begins prior to the patent expiration. However, the PRP Holder may choose to maintain all necessary authorizations and, thus, not exchange information or material. Although these agreements apply to the USA only, they constitute a starting point for the types of obligations that would be expected between a Secondary Party outside the USA and the PRP Holder, in particular in the area of stewardship.

MARKET OPTIONS AND EXAMPLES OF OFF-PATENT UTILIZATION

In the case of the original glyphosate-tolerant Roundup Ready soybean Event, known as GTS 40-3-2, prior to the GEMAA instatement, Monsanto indicated its willingness to maintain full global regulatory support until 2021. Now that GEMAA is active, if Monsanto wants to discontinue the regulatory responsibilities for GTS 40-3-2, it needs to notify all interested parties at least 7 years prior to any such discontinuation. In such a notification, Monsanto, as PRP holder must set forth (i) the discontinuation date and (ii) whether it will retain or transfer the PRP ownership (GEMAA, 2015). In case of discontinuation, it has to announce the last sale. As Monsanto is commercializing a replacement Event for GTS 40-3-2, this Event will logically be discontinued in the future.

After patent expiration, new utilizations may be released for the Off-Patent Event, including for instance saving and replanting seeds of certain varieties in the fields by farmers, provided the originally purchased seeds are not covered by other patents or use restrictions in the seed bag license or in a technology use agreement. Since November 2014, such a use of GTS-40-3-2 has been possible: the University of Arkansas System

Division of Agriculture released a glyphosate-tolerant soybean varieties UA 5414RR in December 2014 and UA 5715GT in April 2016, both available for sale to USA farmers without technology fees and without restrictions on farmer-saved seed (Miller, 2016). Thanks to a specific license from Monsanto, breeding material has been provided to public farmers, including the University of Arkansas (Miller, 2016).

In contrast, in April 2015, Event MON810 conferring insect resistance in corn (*Zea mays)* also became off-patent (GEMAA, 2013), but, since 30 September 2015, its approval by the US EPA as a corn product with a single plant-incorporated protectant has expired and, therefore, cannot be freely used by seed companies and farmers in the USA (U.S. Environmental Protection Agency, 2010). The plant-incorporated protectant in MON810, *Bacillus thuringiensis* Cry1Ab delta-endotoxin, retains an approved status for the US EPA. In this case, because the regulatory approvals for use as a single trait have not been maintained by the PRP Holder after the USA patent expiration, Secondary Parties in the USA have no direct opportunity to exploit the potential of the patent expiration without obtaining of a new permit. Following GTS-40-3-2 and MON810, a handful of Events will also probably become off-patent in the USA between 2014 and 2020, and several more after 2020.

These examples show that the possible use of Off-Patent Events without a large investment in the regulatory package remains very limited for Secondary Parties, because it strongly depends on agreements with the PRP Holders to have access to the Event and to keep approvals in force. Moreover, outside the USA, a contractual framework, such as the AgAccord that would facilitate possibilities for Secondary Parties, is lacking.

EMERGING MARKET OPTIONS AND POTENTIAL DIRECTIONS FOR FURTHER IMPROVEMENT

Many untapped opportunities remain for GM corn in emerging markets: "in Asia, there are about 60 million hectares of potential biotech maize, with 35 million hectares in China alone; there is a similar potential in Africa for up to 35 million hectares of biotech maize" (James, 2016). New GM corn markets in Africa will probably include Nigeria, Ethiopia, Namibia, Swaziland, and Malawi, and Vietnam in Asia. Secondary Parties will hopefully appear in such markets to create new plant breeding and commercial seed activities possibly in their own and the GM maize PRP Holder interests. In these countries as in many other African and South American countries, the patent status of an Event that has been commercialized elsewhere for 20 years is not an issue, because in most of them the Events have not been the subject of patent filings. Consequently, no intellectual property right for the Event exists in these countries and country-dependent patent rights are not extendable to countries where no filing has been done. In many countries, especially in Africa, the biosafety regulatory environment still needs full implementation. In addition, workable seed laws, variety certification procedures, and seed certification schemes are not regionally harmonized and effective, with negative outcomes for breeding investments and for the emergence of professionally certified seed production and reliable seed supplies.

Even if conditions existed favoring the emergence of local and professional seed companies, the African countries willing to regulate the cultivation of GM crops would need to accept the concept of data transportability to facilitate such a development: in agreement with the PRP Holder, data packages establishing the human and environmental safety of the Off-Patent Event agreed in experienced countries, such as South Africa, should be recognized as acceptable in other African countries. In this manner, risk assessment could be focused on studies that analyze the efficacy and environmental impact of the trait under local conditions. When countries have similar growing conditions and pests, data transportability can also apply to field data (Garcia-Alonso et al., 2014). With enhanced internationally harmonized regulatory systems, emerging markets may become the best place for non-conflicting collaborations between PRP Holders and Secondary Parties.

In addition, in the presence of a political willingness, the development of a generic industry for GM crops could be stimulated by initiatives, such as those developed in the pharmaceutical industry following the USA Drug Price Competition and Patent Term Restoration Act (Public Law 98-417). According to this research or safe harbor exemption, performing research and tests for the preparation of regulatory approvals does not constitute infringement for a limited term before the end of the patent term. This exemption allows manufacturers to prepare generic drugs in advance of the patent expiration. In the European Union, equivalent exemptions are allowed.

CONCLUSION

Off-Patent Events for GM crops are and will increasingly become a reality, constituting a major challenge for PRP Holders. By maintaining authorizations, they remain responsible and liable for stewardship and have to keep data updated for regulatory compliance purposes, which is difficult when Secondary Parties use the Off-Patent Event. Although Off-Patent Events utilized as single Events might be scarce, they might be used in combinations with additional traits.

Currently, the GM crop regulatory systems do not facilitate a generic industry for Off-Patent Events. GM regulatory harmonization and simplification, including the acceptance of data transportability among countries and regions, would be a significant achievement for increased use and acceptance of the technology. Such improvements would allow a cost reduction and potentially open the market to new actors, in addition to the few multinationals that currently have the resources to develop and maintain GM Events.

Initiatives, such as the AgAccord are essential to facilitate the further development of Off-Patent Events.

The founding members of the AgAccord could seemingly decide to extend the agreement territory to the rest of the world, without adverse impact on the members, but with many new opportunities for non-USA signatories. As mentioned above, improvements can also be made by taking advantage of the applications in the pharmaceutical industry to speed up the development of generic drugs.

Although emerging markets often still lack a regulatory environment that would allow the commercialization of GM crops, the most promising opportunities for Secondary Parties in direct collaboration with PRP Holders may reside in the African and Asian countries that are in the process of setting up a regulatory framework to handle GM crops for scientific research and for commercialization.

AUTHOR CONTRIBUTIONS

PR, PD, GF, IP, and MH collected the information and wrote the paper.

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GLOSSARY

Event: the unique recombinant DNA insertion event that took place in one plant cell, which was then used to generate a transgenic plant. The selected Event is used for breeding and development of commercial varieties in a crop.

generic: as used here, refers to a product that is not protected by intellectual property rights and that is freely available for use by third parties for commercial and development purposes.

Generic Event: an Event that harbors the same inserted genetic sequences as the corresponding commercialized Event, but that is made *de novo* with nucleotide sequences and technologies available in the public domain.

Off-Patent Event: an existing Event, originally patented by, and commercially available through, a PRP Holder for which the patent protection has expired in a specific territory.

Proprietary Regulatory Property (PRP): The data, dossiers, and authorizations that enable the cultivation and sale of an Event in any countr(y)(ies) in which it is approved for cultivation and allow the importation and use of material containing that Event (seed product, grain, or any product thereof regulated as a result of the Event).

Proprietary Regulatory Property (PRP) Holder: an entity that owns or controls the PRP and any other relevant intellectual property rights for an Off-Patent Event.

Secondary Party: an entity that further develops or uses an Off-Patent Event.

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ORIGINAL RESEARCH
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Assessing the Likelihood of Gene Flow From Sugarcane (*Saccharum* Hybrids) to Wild Relatives in South Africa

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Pre-commercialization studies on environmental biosafety of genetically modified (GM) crops are necessary to evaluate the potential for sexual hybridization with related plant species that occur in the release area. The aim of the study was a preliminary assessment of factors that may contribute to gene flow from sugarcane (*Saccharum* hybrids) to indigenous relatives in the sugarcane production regions of Mpumalanga and KwaZulu-Natal provinces, South Africa. In the first instance, an assessment of *Saccharum* wild relatives was conducted based on existing phylogenies and literature surveys. The prevalence, spatial overlap, proximity, distribution potential, and flowering times of wild relatives in sugarcane production regions based on the above, and on herbaria records and field surveys were conducted for *Imperata*, *Sorghum, Cleistachne,* and *Miscanthidium* species. Eleven species were selected for spatial analyses based on their presence within the sugarcane cultivation region: four species in the Saccharinae and seven in the Sorghinae. Secondly, fragments of the nuclear internal transcribed spacer (ITS) regions of the 5.8s ribosomal gene and two chloroplast genes, ribulose-bisphosphate carboxylase (*rbcL*), and maturase K (*matK*) were sequenced or assembled from short read data to confirm relatedness between *Saccharum* hybrids and its wild relatives. Phylogenetic analyses of the ITS cassette showed that the closest wild relative species to commercial sugarcane were *Miscanthidium capense, Miscanthidium junceum,* and *Narenga porphyrocoma*. *Sorghum* was found to be more distantly related to *Saccharum* than previously described. Based on the phylogeny described in our study, the only species to highlight in terms of evolutionary divergence times from *Saccharum* are those within the genus *Miscanthidium*, most especially *M. capense,* and *M. junceum* which are only 3 million years divergent from *Saccharum*. Field assessment of pollen viability of 13 commercial sugarcane cultivars using two stains, iodine potassium iodide (IKI) and triphenyl tetrazolium chloride, showed decreasing pollen viability (from 85 to 0%) from the north to the south eastern regions of the study area. Future work will include other aspects influencing gene flow such as cytological compatibility and introgression between sugarcane and *Miscanthidium* species.

Keywords: gene flow, hybridization, pollen viability, phytogeography, spatial assessment, phylogeny, *Miscanthidium*

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INTRODUCTION

Commercial sugarcane (*Saccharum* hybrids) was thought to have arisen from an interspecific hybridization event between *S. spontaneum* and *S. officinarum* in Java in the late 1800's (Paterson et al., 2013). Recent literature, though, suggests that the heritage is more complicated, especially when considering the nuclear phyologenetic relationships (Lloyd Evans and Joshi, 2016a). The complex ancestry, the polyploid and aneuploid nature of modern sugarcane makes conventional breeding challenging (Butterfield et al., 2001). Notwithstanding these issues, in excess of 60 "*N*" sugarcane cultivars have been released in the South African industry since 1955, but environmental constraints affect sexual hybridization because floral induction, flowering synchronicity between selected parental germplasm and pollen fertility are problematic at sub-tropical latitudes (Brett, 1950; Horsley and Zhou, 2013). Attempts to increase genetic diversity by intergeneric crossing of commercial hybrids and members of the "*Saccharum* complex" have met with either limited or no success, even under controlled conditions with human intervention, and there are no reports of such hybridization in the wild (Bonnett et al., 2008; Cheavegatti-Gianotto et al., 2011; Organisation for Economic Cooperation and Development, 2013).

Cultivar improvement using genetic modification (GM) technology is being explored and a range of traits have been introduced to sugarcane (reviews by Lakshmanan et al., 2005; Brumbley et al., 2008; Meyer and Snyman, 2013). Commercial cultivation of GM sugarcane has only been approved in Indonesia (Xue et al., 2014) and more recently, Brazil[1], but research of this nature is underway in most sugarcane-producing countries.

In South Africa, legislation governs the use and cultivation of GM crops [namely the Genetically Modified Organisms Act (Act 15 of 1997) and the National Environmental Management Act (Act 107 of 1998)]. One aspect of GM crop cultivation that requires assessment prior to commercial release is establishing the likelihood of lateral gene flow between related plant species. Hybridization is only possible between a crop plant and a wild relative if a number of barriers to gene flow are traversed (McGeoch et al., 2009). According to den Nijs et al. (2004), successful gene transfer (barrier crossing) requires plant populations to: (a) overlap spatially; (b) overlap temporally (flowering periods); and (c) be sufficiently close biologically that the resulting hybrids are fertile, facilitating introgression of genetic material into a new population. The probability of and extent of gene flow varies according to these limiting factors (Légère, 2005).

Gene flow from transgenic crops to wild relatives may have negative environmental effects if the hybrid plants inherit an increased capacity for invasiveness and weediness of a species (e.g., by conferring a trait such as herbicide tolerance to a specific/related active ingredient would be problematic if that was the only mechanism of eradication) (Andow and Zwahlen, 2006). Furthermore, gene flow from GM plants may be difficult to contain, demonstrated by transgene movement in rice (traits such as high protein content, disease and insect resistance and herbicide and salt tolerance), creeping bentgrass (herbicide tolerance), and oilseed rape (herbicide tolerance) (Rieger et al., 2002; Warwick et al., 2003; Chen et al., 2004; Watrud et al., 2004; Zapiola et al., 2008). This could lead to the evolution of highly competitive weeds and the degeneration of the genetic diversity in indigenous grasses.

This study was conducted to assess the likelihood of gene flow from commercial sugarcane to wild relatives in the sugar production regions of South Africa. Factors such as spatial overlap, proximity, flowering synchrony and pollen viability are prerequisites for hybridization to occur. Therefore, if close relatives occur in areas where sugarcane is cultivated, then transgenic sugarcane presents a likelihood for gene flow to these species. To assess this possibility, the objectives are as follows: (i) review the literature to identify the wild relatives of *Saccharum*, collate what is known about gene flow between cultivated *Saccharum* hybrids and wild relatives in South Africa, determine overlapping flowering times and assess pollen viability of commercial sugarcane; (ii) quantify the distribution of wild *Saccharum* relatives and assess the spatial overlap of their distributions with commercial sugarcane plantations; (iii) determine phylogenetic relationships within the *Saccharum* complex to confirm which species are most closely related to cultivated sugarcane; (iv) make an assessment of the likelihood of gene flow potential between related species and cultivated sugarcane.

MATERIALS AND METHODS

Phytogeography of *Saccharum* Wild Relatives in South Africa

Wild relatives which diverged from *Saccharum* <7.3 million years ago (based on chloroplast sequence chronograms) were identified from a global phylogeny based on chloroplast genomes/regions for the Poaceae (Skendzic et al., 2007; Soreng et al., 2015; Lloyd Evans and Joshi, 2016a). Eleven species of the Sorghinae and Saccharinae subtribes of the Andropogoneae were selected for spatial analyses based on their presence within the sugarcane cultivation region of South Africa: four species that belong to Saccharinae and seven to Sorghinae (Organisation for Economic Cooperation and Development, 2013; Fish et al., 2015; Soreng et al., 2015). Grass nomenclature is in accordance with The Plant List (2013).

Herbarium specimens were sourced from 11 South African herbaria. All specimen data were captured and a gap analysis conducted for the study area to identify where insufficient information was available regarding the occurrence of wild relatives. At these sites, sugarcane field margins were examined for the target species, especially at the preferred habitats of sugarcane relatives such as disturbed and waterlogged areas. Collections were made during flowering periods, May to July, of 2016 and 2017. Field data of collected species were recorded and specimens accessioned in the A. P. Goossens Herbarium (PUC) and National Herbarium (PRE). Herbarium distribution records of the new collections were added to the master database to construct a distribution map per species with ArcGIS (student

[1]http://www.isaaa.org/

TABLE 1 | Herbarium accession numbers of the different flowering sugarcane cultivars tested for pollen viability and sampled for genomic DNA extraction in 2016 and 2017.

Sugarcane cultivar	GPS Coordinates	Location of plantation	Herbarium accession no.
2016			
Saccharum hybrid cv N36	25°36′08″ S, 31°33′30″ E	Malelane	PUC 14606
Saccharum hybrid cv N23	25°30′05″ S, 31°26′09″ E	Malelane	PUC 14609
Saccharum hybrid cv N36	25°33′08″ S, 31°56′09″ E	Komatipoort	PUC 14615
Saccharum hybrid cv N14	25°33′04″ S, 31°56′02″ E	Komatipoort	PUC 14616
Saccharum hybrid cv N28	27°28′05″ S, 32°09′02″ E	Jozini	PUC 14617
Saccharum hybrid cv N19	27°28′04″ S, 32°09′02″ E	Jozini	PUC 14620
Saccharum hybrid cv N43	27°26′03″ S, 32°10′00″ E	Jozini	PUC 14621
Saccharum hybrid cv N25	27°26′03″ S, 32°09′09″ E	Jozini	PUC 14622
Saccharum hybrid cv N36	27°24′08″ S, 32°09′05″ E	Jozini	PUC 14626
Saccharum hybrid cv N41	27°25′07″ S, 32°10′19″ E	Jozini	PUC 14628
Saccharum hybrid cv N23	27°23′08″ S, 31°39′08″ E	Jozini	PUC 14629
Saccharum hybrid cv N42	28°43′08″ S, 31°55′01″ E	Empangeni	PUC 14630
Saccharum hybrid cv N26	28°43′33″ S, 31°48′41″ E	Empangeni	PUC 14631
Saccharum hybrid cv N19	28°44′05″ S, 31°54′05″ E	Empangeni	PUC 14632
Saccharum hybrid cv NCo376	29°42′12″ S, 31°02′35″ E	Mount Edgecombe	PUC 14656
2017			
Saccharum hybrid cv N36	25°36′55″ S, 31°33′12″ E	Malelane	PUC 14678
Saccharum hybrid cv N14	25°36′01″ S, 31°33′11″ E	Malelane	PUC 14679
Saccharum hybrid cv N23	25°37′28″ S, 31°32′57″ E	Malelane	PUC 14680
Saccharum hybrid cv N14	25°33′18″ S, 31°55′51″ E	Komatipoort	PUC 14681
Saccharum hybrid cv N36	25°33′40″ S, 31°55′44″ E	Komatipoort	PUC 14682
Saccharum hybrid cv N23	27°25′22″ S, 31°38′35″ E	Pongola	PUC 14683
Saccharum hybrid cv N14	27°24′23″ S, 31°37′33″ E	Pongola	PUC 14684
Saccharum hybrid cv N43	27°29′05″ S, 32°08′51″ E	Jozini	PUC 14685
Saccharum hybrid cv N19	27°28′52″ S, 32°09′30″ E	Jozini	PUC 14686
Saccharum hybrid cv N23	27°26′19″ S, 32°09′59″ E	Jozini	PUC 14688
Saccharum hybrid cv N36	27°26′06″ S, 32°09′55″ E	Jozini	PUC 14689
Saccharum hybrid cv N14	27°26′19″ S, 32°09′59″ E	Jozini	PUC 14690
Saccharum hybrid cv N23	28°26′52″ S, 32°13′38″ E	Mtubatuba	PUC 14691
Saccharum hybrid cv N42	28°28′39″ S, 32°18′32″ E	Mtubatuba	PUC 14693
Saccharum hybrid cv N14	28°29′05″ S, 32°16′18″ E	Mtubatuba	PUC 14694
Saccharum hybrid cv NCo376	28°29′10″ S, 32°15′30″ E	Mtubatuba	PUC 14696
Saccharum hybrid cv N36	28°27′57″ S, 32°18′02″ E	Mtubatuba	PUC 14697
Saccharum hybrid cv N42	28°44′51″ S, 31°55′36″ E	Empangeni	PUC 14698
Saccharum hybrid cv N42	29°28′59″ S, 31°08′14″ E	Umhlali	PUC 14699
Saccharum hybrid cv N27	30°38′01″ S, 30°30′03″ E	Port Shepstone	PUC 14704
Saccharum hybrid cv N42	30°37′59″ S, 30°30′06″ E	Port Shepstone	PUC 14705
Saccharum hybrid cv N58	30°38′01″ S, 30°30′08″ E	Port Shepstone	PUC 14706
Saccharum hybrid cv N36	30°38′02″ S, 30°30′08″ E	Port Shepstone	PUC 14707
Saccharum hybrid cv N39	30°38′57″ S, 30°29′25″ E	Port Shepstone	PUC 14710
Saccharum hybrid cv NCo376	30°38′53″ S, 30°29′15″ E	Port Shepstone	PUC 14711
Saccharum hybrid cv NCo376	29°42′11″ S, 31°02′34″ E	Mount Edgecombe	PUC 14715
Saccharum hybrid cv N42	29°42′12″ S, 31°02′36″ E	Mount Edgecombe	PUC 14716

edition version 10.3, Esri, USA) to confirm their presence in sugarcane cultivation areas (Supplementary Figure 1).

Plant Material

Leaf samples from *Saccharum* hybrid parental breeding lines were collected at SASRI, Mount Edgecombe (23 May 2016). Leaf samples from commercial sugarcane cultivars were collected from grower plantations (4–7 July 2016). Herbarium records and iSpot[2] were used to pinpoint localities and habitat types where selected wild relatives of *Saccharum* have been collected

[2]https://www.ispotnature.org

TABLE 2 | Taxa used for phylogenetic analyses to determine relatedness.

Species name	Geographical origin	Herbarium accession no.	Place of collection (province, place)	Reclassification	Data source or accession number (ITS; *matK; rbcL*)
Saccharum robustum	Papuasia, South-east Asia	PUC 14591	KZN, SASRI nursery	–	–
Saccharum arundinaceum	Papuasia, South-east Asia	PUC 14594	KZN, SASRI nursery	*Tripidium arundinaceum* (Retz.) Lloyd Evans	–
Saccharum hybrid cv Rowan Green	SASRI, RSA	PUC 14598	KZN, SASRI nursery	–	–
Saccharum hybrid cv Co745	SASRI, RSA	PUC 14600	KZN, SASRI nursery	–	–
Saccharum hybrid cv N14	SASRI, RSA	PUC 14614	MP, Komatipoort sugarcane plantation	–	–
Saccharum hybrid cv N36	SASRI, RSA	PUC 14606	MP, Malelane sugarcane plantation	–	–
Saccharum hybrid cv NCo376	SASRI, RSA	PUC 14656	KZN, SASRI germplasm nursery	–	–
Trachypogon spicatus	Southern Africa	PUC 14655	NW, Ikageng roadside	–	–
Sorghum versicolor	Africa	PUC 10278	NW, Potchefstroom roadside	*Sarga versicolor* (Andersson) Spangler	–
Ischaemum afrum	Africa, India	–	–	–	HM347038.1; KU291467.1; KU291467.1
Miscanthus junceus	Southern Africa	–	–	*Miscanthidium junceum* (Stapf) Stapf	SRR3968481; SRR396848; SRR396848
Hemarthria sibirica	India, Temperate East Asia	–	–	–	KF163639.1; KF163806.1; KF163515.1
Sorghum timorense	Asia, Australasia	–		*Sarga timorense* (Kunth) Lloyd Evans	SRR424217; SRR424217; SRR424217
Sorghum versicolor 2	Tropical and Subtropical Africa	–		*Sarga versicolor* (Andersson) Spangler	SRR427175; SRR427175; SRR427175
Miscanthus capensis	Southern Africa	–	–	*Miscanthidium capense* (Nees) Stapf	BeauSci; BeauSci; BeauSci
Saccharum narenga	Temperate and Tropical Asia, Ethiopia	–		*Narenga porphyrocoma* (Hance ex Trimen) Bor	SRR3399436; SRR3399436; SRR3399436
Saccharum spontaneum SES234B	North Africa, India, Temperate and Tropical Asia, Papuasia	–		–	SRR486146; LN849912.1; LN849912.1
Saccharum spontaneum SES196	North Africa, India, Temperate and Tropical Asia, Papuasia	–		–	SRR2899231; SRR2899231; SRR2899231
Saccharum sinense Tekcha	India, China	–		–	SRR2891264; SRR2891264; SRR2891264
Saccharum hybrid cv SP80-3280	Brazil	–		–	SRR1774133; SRR1774133; SRR1774133 HM347038.1; KU291467.1; KU291467.1
Saccharum officinarum IJ76-514	Papuasia	–		–	SRR528718; LN849913.1; LN849913.1 SRR3968481; SRR396848; SRR396848
Saccharum robustum NG57-054	Papuasia	–		–	SRR2899233; SRR2899233; SRR2899233 AF190756.1; LN906656.1; KR737308.1
Miscanthus sacchariflorus Hercules	Temperate north-east Asia	–		–	BeauSci; BeauSci; BeauSci

(Continued)

TABLE 2 | Continued

Species name	Geographical origin	Herbarium accession no.	Place of collection (province, place)	Reclassification	Data source or accession number (ITS; *matK; rbcL*)
Miscanthus oligostachyus	Temperate Asia	–		–	HQ822027.1; BeauSci; BeauSci
Miscanthus floridulus US56-0022-03	India, Asia Tropical, Asia Temperate, Melanesia, Papuasia, Polynesia	–		–	SRR486154; SRR486154; SRR486154 DQ005089.1; KU556663.1; KP996860.1
Miscanthus sinensis Andante	Caucasus, Indo-China, East Asia, Malesia, Australasia, Continental America (introduced)	–		–	BeauSci; BeauSci; BeauSci AF190756.1; LN906656.1; KR737308.1
Polytoca digitata	Africa, Triopical Asia, Temperate Asia, Papuasia, Malesia	–		–	GQ870232.1; KY596178.1; KY596178.1
Polytrias indica	Tropical west-central Africa, Temperate Asia, China, Malesia, Papuasia, Australasia, Polynesia, South America, Mesoamerica	–		–	GQ870228.1; —; —
Microstegium vimineum 2	Africa west-central Tropical, Caucasus, Temperate Asia, Tropical Asia, Indo-China, Malesia	–	–	–	ERR2040772; ERR2040772; ERR2040772
Bothriochloa insculpta	Southeast Europe, Africa, Macaronesia, Arabia, Temperate Asia, Tropical Asia, Australasia	–	–	–	AF190756.1; MF963585.1; MF963222.1
Andropogon glomeratus var *scabriglumus*	Americas	–		–	MF964041.1; MF963585.1; MF963222.1
Andropogon virginicus	Caucasus, Temperate Asia, Australasia, Continental America	–		–	BeauSci; BeauSci; BeauSci
Hyparrhenia rufa	Africa, India, Temperate Asia, Tropical Asia, Malesia, Papuasia, Australasia, Continental America	–	–	–	GQ870187.1; KY596156.1; KY596156.1
Schizachyrium sanguineum	Tropical Africa, India. China, Temperate Asia, Malesia, Papuasia, Americas	–	–	–	DQ005070.1; KY596124.1; KY596124.1
Cymbopogon flexuosus	Eastern Africa, Temperate Asia, Tropical Asia, Malesia, Papuasia	–		–	SRR2970609; SRR2970609; SRR2970609
Sorghastrum nutans	Australasia, North America	–		–	DQ005080.1; KU291482.1; KU291482.1
Sorghum ×drummondii	Central, Eastern and Southern Europe, Eastern and Southern Africa, Malesia, Asia, Continental America	–		–	SRR998968; SRR998968; SRR998968
Sorghum arundinaceum 2	North Africa, Macronesia, Tropical Asia, India, Papuasia, Australasia	–		–	SRR999026; SRR999026; SRR999026
Sorghum halepense 2	Central, Southern and Eastern Europe, North Africa, Macronesia, Temperate Asia, Tropical Asia, Malesia, Papuasia, Australasia	–		–	SRR486216; SRR486216; SRR486216 HM347038.1; KU291467.1; KU291467.1

(Continued)

TABLE 2 | Continued

Species name	Geographical origin	Herbarium accession no.	Place of collection (province, place)	Reclassification	Data source or accession number (ITS; *matK; rbcL*)
Sorghum propinquum 369-1	Temperate Asia, Tropical Asia, Malesia, Papuasia, Pacific	–		–	SRR998982; SRR998982; SRR998982 SRR3968481; SRR396848; SRR396848
Germainia capitata	Temperate China, Tropical Indo-China, Australasia, Papuasia	–		–	GQ870198.1; KY596175.1; KY596175.1 AF190756.1; LN906656.1; KR737308.1
Microstegium japonicum	Temperate Asia, Caucusus, Eastern Asia	–		–	KF163847.1; KF163826.1; KF163826.1
Microstegium nudum	Southern Africa, Tropical Africa, Temperate Asia, Tropical Asia, Malesia, Papuasia, Australasia	–		–	EU489073.1; —; MF998299.1
Saccharum arundinaceum 2	Temperate Asia, Tropical Asia, India, Malesia, Papuasia	–		*Tripidium arundinaceum* (Retz.) Lloyd Evans	BeauSci; SASRI; SASRI DQ005089.1; KU556663.1; KP996860.1
Saccharum ravennae	Temperate Asia, Tropical Asia, India, Malesia, Papuasia	–		*Tripidium ravennae* (L.) H. Scholz	[AF019824.1/AY116296.1]; SASRI; SASRI DQ005089.1; KU556663.1; KP996860.1
Imperata cylindrica	Southwestern Europe, North Africa, East Africa, Southern Africa, Temperate Asia, Tropical Azsia, Malesia, Papuasia, Australasia	–			SRR4280862; SRR4280862; SRR4280862 AF190756.1; LN906656.1; KR737308.1
Tripsacum dactyloides	Mesoamerica, Caribbean, Australasia	–		–	SRR5127199; SRR5127199; SRR5127199
Zea mays B73	Mesoamerica	–		–	SRR447986; KF241981.1; KF241981.1
Sorghum bicolor BTx623	North, East and West African in origin, globally distrubuted	–		–	SRR3923525; EF115542.1; EF115542.1 DQ005089.1; KU556663.1; KP996860.1
Sorghum laxiflorum	Tropical Asia, Malesia, Papuasia, Australasia	–		–	AF019824.1; —; — DQ005089.1; KU556663.1; KP996860.1
Cleistachne sorghoides	Tropical and Subtropical Africa, Arabia, Tropical Asia, Temperate Asia	–		–	U04790.1; —; —

For data sources, all entries beginning with SRR or ERR were downloaded from NCBI's sequence read archive and sequences were assembled as SASRI. Sequences labeled BeauSci or SASRI were either gifted by BeauSci Ltd. or were within the SASRI short read collection. Unlabelled sequences were collected and sequenced at SASRI. All other sequences were downloaded from the NCBI nucleotide archive. The symbol — in the accession no. column indicates that sequence information was not available. Where there are two GenBank accessions for a sequence, this indicates that these sequences were merged prior to analysis—shown in []. NW—North West Province, RSA; KZN—KwaZulu-Natal Province, RSA; SASRI—South African Sugarcane Research Institute, Mount Edgecombe, KZN, RSA.

in the past and are known to occur. Samples of plant leaf material were collected from these locations, for which plant specimens are deposited in the A.P. Goossens Herbarium (PUC) (**Table 1**). The leaf material was decontaminated with 70% (v/v) ethanol and stored in 50 ml plastic tubes (Thermo Scientific Group) filled with 15 g silica gel. Related species and outgroups that could not be collected in the field were sourced from GenBank genetic sequence database (**Table 2**).

DNA Extraction, Amplification, and Sequencing

Between 0.10–0.15 g of dry plant leaf material per species was homogenized in liquid nitrogen and genomic DNA was isolated (GeneJET Plant Genomic DNA Purification kit; Thermo Fisher Scientific, USA) according to the manufacturer's protocol. The purity and concentration of the DNA was assessed (NanoDrop ND-1000 spectrophotometer; NanoDrop Technologies, Inc., Thermo Scientific Group).

DNA sequences of the internal transcribed spacer (ITS) regions of the 5.8s ribosomal gene as well as that of two chloroplast genes, ribulose-bisphosphate carboxylase (*rbcL*) and maturase K (*matK*) were used to design primers (**Table 3**). Amplification of the above three regions was done via Polymerase Chain Reaction (PCR) on a C1000 Thermal Cycler (BioRad, USA). The reaction mixture included 2X KAPA Taq readyMix PCR kit (1x PCR buffer, 2 U Taq DNA polymerase, 0.2 mM of each DNTP, 1.5 mM $MgCl_2$ and stabilizers), 0.5 μM forward and reverse specific primers, 5–50 ng DNA template and nuclease-free water. For each primer set (**Table 3**) the initial denaturation step was at 94°C for 3 min, followed by denaturation at 94°C for 60 s. Annealing temperatures varied depending on the primer set: 50°C for 30 s (ITS and *rbcL*) and 48°C for 40 s for *matK*.; the extension step was at 72°C for 30 s (ITS and *rbcL*) and 60 s for *matK*. There were 35 thermocycles for ITS and *rbcL* and 40 for *matK*. The final extension step was at 72°C for 10 min. PCR products were visualized on a 1% (w/v) agarose gel and cleaned-up (GeneJET PCR purification kit; Thermo Fisher Scientific, USA).

Sequencing reactions were performed with the same primers as those used for PCR using the BigDye Terminator V1.3 cycle sequencing kit (Applied Biosystems, USA). This was followed by fluorescence-based DNA analysis using capillary electrophoresis technology on the Applied Biosystems 3500 Genetic Analyser. Sequences were analyzed and trimmed using Sequencing Analysis V5.3.1 (Applied Biosystems).

Sequence Assembly

The 5.8s genomic ITS cassette along with the chloroplastic *matK* and *rbcL* genes were chosen for phylogenetic analysis. In those cases where no ITS, *matK,* or *rbcL* sequences could be found in GenBank, sequences were assembled from short read data (either mined from NCBI's SRA archive[3] or made available through on-going collaborations) (**Table 2**) using a bait-and-assemble assembly method described previously (Lloyd Evans and Joshi, 2016b). Third party data assembled for this study are noted in **Table 2** and the assemblies are provided as Supplementary File 1.

Sequence Alignments

The ITS cassette (18s rRNA partial, ITS1 complete, 5.8s rRNA, ITS2 complete, 28s rRNA partial) region was aligned as described previously (Martin et al., 2017). Briefly, DNA sequences (**Table 2**) were aligned with SATÉ (Liu et al., 2009) using MAFFT (Katoh and Standley, 2013) as the aligner, MUSCLE (Edgar, 2004) as the sub-alignment joiner and RAxML as the tree estimator. The final RAxML tree was used as input for PRANK (Löytynoja et al., 2012) an indel-aware alignment optimizer. PRANK was run for 5 generations, using RAxML (identifying the most likely tree from 100 samples) for Maximum Likelihood (ML) tree estimation until both the alignment and the tree topology stabilized. The chloroplastic *matK* and *rbcL* sequences were aligned with SATÉ.

Long-branch attraction and incomplete sampling (Philippe et al., 2017) can be major confounding effects in phylogenetic inference. In an attempt to minimize these effects, at least two exemplars for each sequence were included in the initial alignment and as many species and genera were sampled as possible. To test for long-branch attraction a custom PERL script was written. This script removed one sequence at a time from the final alignment. The reduced alignment was analyzed with RAxML where the most likely tree was identified from 100 random replicates. After the analysis, all trees were compared and where the initial reference tree and the resampled tree differed significantly the deleted sequence was labeled as responsible for long-branch effects and was removed from all subsequent analyses. The sequences remaining after this test were re-aligned using SATÉ and PRANK, as described above. These sequences yielded the final alignment. The final ITS alignment and phylogeny along with the *matK* alignment and phylogeny and the *rbcL* alignment and phylogeny were deposited in TreeBase[4].

Wherever possible, the entire ITS cassette was used. However, where no alternate data was available, the shorter assemblies from existing sequence data were integrated into the alignment and padded with Ns.

Partition Analyses

The ITS cassette was divided into 18s rRNA, ITS1, 5.8s rRNA, ITS2, and 28s rRNA regions, whilst the entire *matK* and *rbcL* genes were analyzed as a single partition. Best-fit evolutionary models were determined using jModelTest2 (Darriba et al., 2012) under the AICc criterion. The best fit models were found to be: 18s RNA: TVM + G; ITS1: TPM3uf + G; 5.8s rRNA: JC + G; ITS2: GTR + G; 28s rRNA: GTR + G; *matK*: TVM + G; and *rbcL*: HKY + I.

Phylogenetic Analyses

Phylogenetic analyses were run for the ITS cassette along with separate analyses for *matK* and *rbcL*. Non-parametric bootstrap tests (using the above partitioning schema) and SH-aLRT analyses were run with IQ-Tree (Nguyen et al., 2015). Neighbor-Joining analyses were run with APE (Paradis et al., 2004). Bayesian Inference (BI; again using the above partitioning schema) was run with MrBayes (Ronquist and Huelsenbeck, 2003). IQ-Tree analyses were run for 2,000 replicates. MrBayes analyses were run with 50,000,000 generations with sampling every 100th tree. Two independent MrBayes analyses, each of two independent runs, were conducted. To avoid any potential over-partitioning of the data, the posterior distributions and associated parameter variables were monitored for each partition using Tracer v 1.6 (Rambaut et al., 2017). High variance and low effective sample sizes were used as signatures of over-sampling. Burn-in was determined by topological convergence and was judged to be sufficient when the average standard deviation of split frequencies was <0.002 along with the use of the Cumulative and Compare functions of AWTY (Nylander et al., 2008). The first 30% of sampled trees were discarded as burn-in.

Phylogenetic analyses (ML and BI) were summarized with Sumtrees (Sukumaran and Holder, 2010) prior to drawing with FigTree (2017) and finishing with Adobe Illustrator to generate

[3]https://ncbi.nlm.nih.gov/sra

[4]http://purl.org/phylo/treebase/phylows/study/TB2:S22812

TABLE 3 | The primers used for the amplification and sequencing of the internal transcribed spacer (ITS), ribulose-bisphosphate carboxylase (*rbcL*), and maturase K (*matK*) gene fragments used as the basis for the phylogenetic analyses.

Primer name	5′-3′ primer sequence	Size (bp)	References
*rbcL*a-F	ATG TCA CCA CAA ACA GAG ACT AAA GC	±550	Kress et al., 2009
*rbcL*a-R	GTA AAA TCA AGT CCA CCR CG		
ITS 4F	TCC TCC GCT TAT TGA TAT GC	±650	White et al., 1990; Stanford et al., 2000
ITS 5A	CTT TAT CAT TTA GAG GAA GGA G		
matK 472F	CCC RTY CAT CTG GAA ATC TTG GTT	±750	Yu et al., 2011
matK 2148R	GCT RTR ATA ATG AGA AAG ATT TCT GC		

F, forward; R, reverse.

publication-quality figures. The ITS only, *matK,* only and *rbcL* only tree topologies were deposited in TreeBase[4].

Chronogram Generation With r8s

The application r8s (Sanderson, 2003) was employed for chronogram generation. An optimal tree topology was generated and was used for analysis. Parameters were adjusted for ML branch lengths on all trees and divergence timings were estimated with a smoothing factor of 100, the Penalized Likelihood method using the Truncated Newton optimization framework with analytical gradients generated by r8s. To generate 95% confidence intervals on branch times, the non-parametric bootstrap trees generated by IQ-Tree were used as input to r8s. All trees were concatenated into a single nexus file using a custom PERL script and an r8s block was appended so that r8s could be executed over all trees with parameters as defined above. The *profile* command of r8s was employed to individually summarize the distribution of ages at all given nodes of the tree (employing a custom PERL wrapper). Priors for the main nodes were defined as follows: root, fixed age of 13.8 million years ago, *Tripsacum–Germainia* node, fixed age of 9.2 million years ago (Estep et al., 2014), *Sarga–Miscanthidium* node, minimum age of 7.4 million years ago, *Miscanthus–Miscanthidium* node fixed age 3.4 million years ago, *S. spontaneum–S. sinense* node, minimum age of 1.4 million years ago (Lloyd Evans and Joshi, 2016a). All other nodes were unconstrained.

Pollen Viability Testing

Pollen samples from commercial sugarcane cultivars were collected during the flowering season (July 2016 and 2017) from nine different sites in South Africa, two in Mpumalanga and seven in KwaZulu-Natal (**Figure 1**). Sites 1–5 are situated in the irrigated region while sites 6–9 are rain-fed. Fresh pollen was collected from anthers in dehiscence, from three separate inflorescences per cultivar per site. Inflorescence collection was between 6.00 and 8.30 h and viability tests conducted in the field immediately thereafter (Amaral et al., 2013).

Two stains were used to estimate pollen viability: 2,3,5-triphenyl tetrazolium chloride (TTC) (Soares et al., 2013) and iodine potassium iodide (IKI) (Huang et al., 2004). Pollen grains were stained with IKI [1% (w/v) iodine and 2% (w/v) potassium iodide in distilled water] for 5 min, while those stained with TTC [1% (w/v) TTC and 5% (w/v) sucrose in distilled water] were examined after 15 min of incubation in direct sunlight. Viewing was under a compound microscope (Model 11, Wild, Heerbrugg Switzerland) at 100 × magnification and counting was aided using a grid stuck to the underside of each glass slide. A random count of a minimum of 100–150 pollen grains was performed for each cultivar replicate, and the percentage viability was determined as the ratio of viable pollen grains (intense dark color for IKI and deep pink for TTC) divided by the total number of grains.

An average from three pollen counts per cultivar per locality was used for calculating percentage pollen viability. All statistical analyses were carried out using Statistica (version 13; Dell Inc., USA). The Kolmogorov-Smirnoff and Lilliefors tests for normality showed that the data did not meet the assumptions of normality in the distribution of all variables. Therefore the Kruskal-Wallis analysis of variance (ANOVA; non-parametric statistics) for comparing multiple independent groups was used to determine differences between determinants measured.

Environmental data including relative humidity, soil water content at 100 mm depth, minimum and maximum temperatures were extracted from the SASRI weather web[5]. Automatic weather stations were situated at each of the sampling sites. Data was extracted from the first of May 2016 and 2017 up to the day at which sampling took place for each of the sites. Mean values were used for each environmental variable at each site. Day length data with the same time resolution and period was obtained online[6]. The non-parametric Spearman rank correlation coefficient was calculated as a measure of correlation between all possible pairs of variables and significance was tested at the 0.05 level.

Desk-Top Study of Hybridization

Prominent literature was consulted to assess gene flow potential. Printed evidence of reproductive compatibility and the formation of hybrids between commercial sugarcane with target related species were used to assess the likelihood of hybridization. The numbers of publications which reported hybridization were recorded. Successes were scored if the publications reported formation of hybrid progeny (FitzJohn et al., 2007; McGeoch et al., 2009; Organisation for Economic Cooperation and Development, 2013) and ranked accordingly. In cases where

[5]http://www.sasa.org.za/sasri
[6]http://www.timeanddate.com

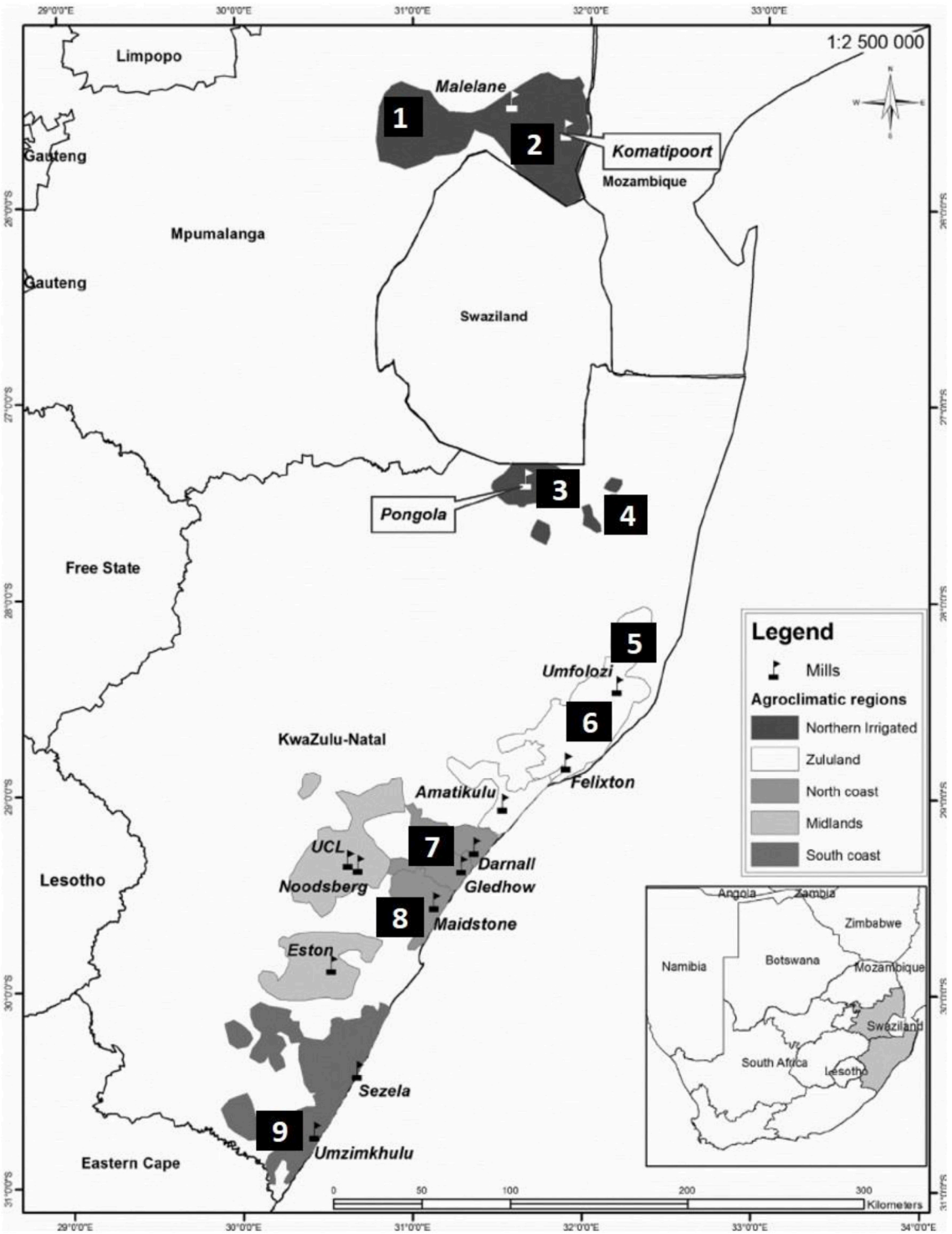

FIGURE 1 | Sugarcane production regions and locations of sugar mills in the Mpumalanga and KwaZulu-Natal provinces of South Africa. Sites for pollen collection were as follows: 1: Malelane; 2: Komatipoort (Mpumalanga); 3: Pongola; 4: Jozini; 5: Mtubatuba; 6: Empangeni; 7: Umhlali; 8: Mount Edgecombe; and 9: Port Shepstone (KwaZulu-Natal).

literature recorded hybridization evidence between *Saccharum* hybrids and wild relatives, the following approaches were undertaken: (i) if target species were reported to hybridize with *Saccharum* hybrids, the number of publications and successes were recorded and scored 1 per event; (ii) if species not found in South Africa hybridized with *Saccharum* hybrids, and the

genus is present in the sugar production area, the species from such genera were treated as reproductively compatible with commercial sugarcane and the number of publications and successes recorded and scored 0.5 per event. The wild relative-*Saccharum* crosses with most hybrids ranked the highest and species with fewer hybrids were ranked lower.

Flowering Times

Flowering times were assessed using literature, herbarium specimens and collections made during field surveys. *Saccharum* hybrids flower from March to August in South Africa (Sithole and Singels, 2013; Zhou, 2013). Plant specimens with inflorescences, dates of collections and occurrence in the study area were used to analyse flowering times in addition to collections sampled during the study. The overlapping percentages between the flowering time of *Saccharum* hybrids and each wild relative was calculated by dividing the number of overlapping months with the total number of months of sugarcane flowering. The wild relatives with more overlapping months were ranked the highest and species with less overlap were ranked lower.

Spatial Assessment

The qualitative assessment to determine the likelihood of wild relatives co-occurring with cultivated sugarcane, which may enhance gene flow potential, was based on the following factors: prevalence, spatial overlap, proximity, distribution potential, gene flow potential, and flowering times (Ellstrand et al., 1999; Chapman and Burke, 2006; Schmidt and Bothma, 2006; Tesso et al., 2008; McGeoch et al., 2009; Andriessen, 2015). All target species were assessed and ranked per factor, whereby species with highest rank was scored 11 and species with lowest rank was scored 1. In the cases where no information was available for a species, the species could not be ranked and was scored 0 (no evidence equates to no ranking). It would be inaccurate to rank species without data, as it would inflate the likelihood scores for the areas where these species were found.

Sugarcane production areas for Limpopo, Mpumalanga and KwaZulu-Natal were obtained from the 2015 National Land Cover dataset. These areas were then overlaid with a grid of quarter-degree squares (QDS) using ArcGIS to provide 113 mapping units for the spatial assessment (Robertson and Barker, 2006). Some of these QDS overlap with Mozambique and Swaziland, but no data was available for these areas. It should be noted that wild relatives may be present in those jurisdictions and did not form part of this study.

The presence of wild relatives in QDS of sugarcane cultivation areas were used to calculate their prevalence, i.e., how common these species are in the study area. The number of individuals per species per QDS within the sugarcane cultivation area was determined. The proportion of individuals per species within QDS was calculated. The same procedure was followed for QDS bordering sugarcane cultivation areas. These proportions were summed to determine the proportional prevalence of each species in the study area. These prevalence values were then sorted from highest to lowest proportion of individuals per species within and bordering sugarcane QDS and scored.

Spatial overlap is the notion of similarity in distribution patterns (or shared occurrences). It was calculated for each species by dividing the number of QDS that overlap with sugarcane cultivation areas with the total number of QDS for sugarcane cultivation areas. This derived a percentage of overlap per species. Species were ranked from highest to lowest based on overlap percentage, with the highest rank scoring 11 and lowest rank scoring 1.

Pollen of graminoids can travel up to 700 m from the donor plant (Schmidt and Bothma, 2006). This was set as the cut-off for proximity measures both during field work and extracting data from herbarium specimens. The herbarium record database was used to construct a table of habitat notes per species and the presence or absence of wild relatives in the vicinity of sugarcane fields were noted. These records were combined with confirmations from the literature and field surveys. Species with more occurrences within the 700 m zone (high proximity) were ranked higher than species with few or no records in sugarcane fields and margins.

Weedy grasses are often spread by different modes of transport (Milton, 2004). Transport networks therefore gives an indication of the potential for weedy relatives of sugarcane to spread, with denser networks implying higher chances for migrations. Road and railway networks were used to calculate the spatial distribution potential of wild relatives across the study area. For each species the number of railway lines and roads per QDS were counted respectively. Totals of QDS containing railways and roads per species were summed. Higher totals were considered indicative of a wild relative's ability to disperse and ranked as highest likelihood for the species to spread to sugarcane fields (Knispel et al., 2008).

Likelihood Scores

Likelihood scores were calculated per species to determine which *Saccharum* relatives might present a higher likelihood for gene flow with sugarcane based on relatedness, flowering time and spatial assessment. Factors were weighted equally for relatedness and spatial assessments (Butler et al., 2007). Relatedness was calculated from the phylogenetic classification and hybridization events, and spatial assessment involved prevalence, spatial overlap, proximity, and distribution potential. Thereafter, spatial, temporal (flowering time) and relatedness assessments were weighted 1:1:2 to come up with a final likelihood score. This weighting was based on the assumption that gene flow and relatedness are not correlated due to reproductive barriers such as flowering time (Panova et al., 2006), and that gene flow likelihood is evenly dependent on temporal and spatial assessment factors. Relatedness is weighted more as it becomes the determining factor for gene flow when prevalence, spatial overlap, proximity, distribution potential or flowering time provide the required compatibility for pollen from one species to reach the stigma of another species.

Likelihood maps indicating various levels of potential for gene flow to occur between *Saccharum* hybrids and wild relatives within sugarcane production areas of eastern South Africa was constructed based on the factor scores per species and summed per grid. The following classes were used for assessing the

likelihood for gene flow: *Sorghastrum nudipes* scored 6 and there was no sugarcane QDS containing only this wild relative species. QDS with sugarcane plantations without wild relatives (0–12); sugarcane QDS plantations with wild relatives: very low (13–43); low (44–86); high (87–129); very high (130–172).

RESULTS

Assessing Hybridization Potential From the Literature

A literature review of hybridization events between cultivated sugarcane and its relatives, revealed 39 hybridization incidents were reported in 23 different studies dating from 1935 to 2014 (reviews by Bourne, 1935; Gao et al., 2014). From these, there were only three claims of spontaneous hybridization (Parthasarathy, 1948; Ellstrand et al., 1999), with the remaining crosses requiring human intervention in artificially controlled conditions using experimental procedures that maximized flowering, pollination and seedling survival. Crosses were performed to integrate the beneficial traits of one species to another to enhance agronomic traits such as growth, ratoonability and biomass accumulation (Brett, 1950; Piperidis et al., 2000; Aitken et al., 2007; Gao et al., 2014).

The genus previously known as *Erianthus* (now divided into *Tripidium* and *Saccharum*) was utilized in 18 of the artificial man-made crosses, predominantly with *Saccharum arundinaceum* (synonym *Erianthus arundinaceus, Tripidium arundinaceum*). Similarly, the number of crosses made with cultivated sugarcane was mainly with the *Saccharum* genus (10 crosses) and with *S. arundinaceum* (4 crosses). Other genera which have been crossed with sugarcane include *Bambusa*, *Imperata*, *Miscanthidium*, *Sorghum,* and *Zea*. Of the 18 species that have been involved in hydridization with sugarcane, seven occur in South Africa and comprise 30.77% of the total hybridization events. The highest number of seedling survival in cultivation was 1,371, resulting from *Saccharum* hybrids × *Sorghum bicolor* (L.) Moench, representing a 9.7% recovery rate from 14,141 total seedlings produced from the crosses (Hodnett et al., 2010). The lowest seedling survival was from a cross involving *Zea mays* L., where only one from more than 1,000 seedlings survived (Bonnett et al., 2008). One of the reported crosses involving *S. bicolor* failed with no true seedlings obtained (Bourne, 1935). With the exclusion of the former attempt, 48.72% studies used molecular markers to verify the presence of the maternal and paternal alleles from putative hybrids, whereas the remaining crosses (51.38%) relied on visual inspection of inherited morphological characteristics against those of parent lines as well as chromosome counts (Khanyi, 2018).

Imperata cylindrica, *Sorghum arundinaceum*, *S.* ×*drummondii,* and *S. halepense* were the only species that were found to be reproductively compatible with *Saccharum* species based on assessed literature (**Table 4**). *Miscanthidium capense* and *Miscanthidium junceum* were not part of any species-specific hybridization studies, but were scored as compatible reproductive species based on the literature reporting on other species of the genus hybridizing with *Saccharum* species (**Table 4**). *Miscanthidium* hybridization is especially documented in the literature (17 publications) of which six reported successes. Hybridization potential between *Miscanthidium* and *Saccharum* ranked highest, *I. cylindrica* was reported in five publications with one success and *S. halepense* was recorded in two publications with one success (**Table 4**). There were considerably more publications on other *Sorghum* species hybridizing with *Saccharum* species, which was not included in the analyses due to uncertainty regarding the generic divisions within the *Sorghum* complex.

TABLE 4 | Summary of gene flow reports between *Saccharum* hybrids and wild relatives from the literature for genera present in the sugarcane cultivation areas.

Species	No. publications reporting hybridization	No. reports of successful hybridization	Success %	Score
Cleistachne sorghoides	–	–	0	0
Imperata cylindrica	5	1	20	7
Microstegium nudum	–	–	0	0
Miscanthidium spp.	9	3	33	8
Sarga versicolor	–	–	0	0
Sorghastrum nudipes	–	–	0	0
Sorghastrum stipoides	–	–	0	0
Sorghum arundinaceum	1	1	100	11
Sorghum ×*drummondii*	1	1	100	11
Sorghum halepense	2	1	50	9

Rankings were based on the number of successful hybridization events, with the highest ranking scoring 11. A score of 0 was given when no instances of hybridization were reported in the literature and therefore no gene flow risk is currently known (no evidence equates to no ranking). Miscanthidium was treated at species level as hybridization was not conducted with species found in South Africa.

Occurrence of Andropogoneae in Sugarcane Cultivation Areas

A total of 815 herbarium specimens of 11 *Saccharum* wild relative species were sourced from 11 herbaria. These records were supplemented by 34 observations of *Saccharum* wild relatives during field visits to sugarcane cultivation areas in South Africa. All 11 wild relatives of the Andropogoneae have been recorded from sugarcane cultivation areas. Six species occurred throughout the sugar cultivation region, but *M. capense* (previously *Miscanthus capensis*), *Sorghum* ×*drummondii,* and *Sorghastrum stipoides* were restricted to the southern parts, and *Cleistachne sorghoides,* and *S. nudipes* to the northern parts of the cultivation area.

Pollen Viability of Commercial Sugarcane Cultivars

A total of 11 sugarcane cultivars were tested for pollen viability during 2016 from six sites in the study area. Pollen viability tests during 2017 included two additional cultivars, N39 and N58, from site 9. No significant difference in pollen viability using two stains, IKI (40.5%) and TCC (38.1%), was observed when comparing 42 individual counts (Kruskal-Wallis ANOVA; $p =$

0.622), therefore results presented are those obtained using the TTC stain for 2016 and 2017 (**Figures 2A,B**, respectively).

For both years, 2016 and 2017, the highest mean percentage viability was observed in cultivar N36 (62.5 and 84.6%, respectively), followed by N14 (46.2 and 83.8%, respectively) in the northern irrigated regions of Mpumalanga. Pollen from all the other cultivars (N19, N23, N25, N27, N28, N41, N42, N43, and NCo376) during the same year had lower mean percentages of viability ranging from 0 to 7.6%, while pollen from N23, N42, N58, and NCo376 was not viable in 2017. In 2017, pollen viability decreased from 84.6% in the northern irrigated regions (site 1) to 0% in the southern rain-fed coastal regions of the study area (site 9) (**Figure 2**), likely due to less favorable environmental conditions. None of the sites had optimal conditions required for flowering (reviewed by Cheavegatti-Gianotto et al., 2011; Organisation for Economic Cooperation and Development, 2013), but percentage pollen viability had a significant positive correlation with both mean maximum temperature ($r = 0.6$) and day length ($r = 0.5$), and a significant negative correlation with soil water content ($r = -0.4$) (results not shown). It must be noted that different cultivars were planted at the sampling sites.

Flowering Times

Information sourced from herbarium labels and field surveys highlighted that *I. cylindrica* and *S. arundinaceum* flower throughout the year, suggesting a 100% flowering synchrony with *Saccharum* hybrids (**Table 5**). *Miscanthidium capense* has an 83% overlap in flowering time with *Saccharum* hybrids. More than 66% of flowering synchrony was further depicted for *Microstegium nudum, M. junceum, S.* ×*drummondii,* and *S. halepense* (**Table 5**).

Determining Genetic Relatedness Using Phylogenetic Analyses

The initial experimental design was based on chloroplast phylogenies. However, during the course of the study, the paper of Folk et al. (2017) highlighted the importance of ancient reticulate evolution and parallel organellar capture in plant evolution. As a result of that paper, we performed an ITS-based phylogeny to check for reticulate evolution in the Andropogoneae. The overall ITS cassette phylogeny (**Figure 3**) is consistent with previous genomic studies of the Andropogoneae (Estep et al., 2014; Welker et al., 2015). However, we have increased resolution of the core Saccharinae and from our analyses, *Saccharum sensu stricto* (*Saccharum spontaneum* and its sister group) is sister to *Miscanthidium* and *Narenga* with good support. This crown group is in turn sister to *Miscanthus* (with moderate support). The entire grouping is, in turn, sister to *Sarga* (with moderate support).

In common with the findings of Hodkinson et al. (2002) we also see *Polytoca digitata* within this grouping. *Microstegium* is clearly not monophyletic and we place *Microstegium vimineum* (with good support) as an outgroup to the entire clade that might be described as the "Saccharinae." The core Andropogoneae is sister to the Saccharinae and *Sorghum* is placed as sister to the core Andropogoneae, although with only moderate support (73% SH-aLRT and 0.8 BI). Though the support for the placement of *Sorghum* is not strong, all independent tree topologies (SH-aLRT, maximum likelihood and Bayesian inference) agree on the topology and our placement of Sorghum as sister to the core Andropogoneae is consistent with the work of Hawkins et al. (2015) who analyzed multiple genes. This confirms the presence of reticulate evolution in the origins of Andropogoneae and casts doubt on many conclusions determined from chloroplast only datasets.

Of the two chloroplastic genes chosen for this study, *matK* provided only a relatively weak phylogenetic signal with over 50% of sequences undetermined and *rbcL* provided no phylogenetic signal (data submitted to TreeBase). Both chloroplastic genes failed IQ-Tree statistical testing for phylogenetic signal. Moreover, as the chloroplastic signal for many of the genera (particularly *Imperata* and *Sorghum*) differ (compare: Estep et al., 2014; Hawkins et al., 2015 and Burke et al., 2016) combining genomic (ITS) and chloroplastic (*matK* and *rbcL*) data would be detrimental to the overall topology of the phylogeny, particularly as genomic data is currently considered to present the "true" evolutionary signal (Estep et al., 2014).

The Maximum Likelihood phylogeny was converted into a chronogram (**Figure 4**) using r8s (Sanderson, 2003) with 95% branch confidence values determined by re-analyzing the non-parametric bootstrap tree set generated by IQ-Tree. Broadly, timings are consistent with previous work (Estep et al., 2014; Lloyd Evans and Joshi, 2016a) with only the genera *Miscanthus* and *Miscanthidium* lying within the 3.4 million year window where wild hybridization is possible as determined by Lloyd Evans and Joshi (2016a) when analyzing wild (i.e., not human mediated) hybridization within the Andropogoneae, specifically the Saccharinae. As it is placed within *Sarga*, *C. sorghoides* is the only other South African genus (apart from *Miscanthidium*) that lies within the 7.4 million year window chosen as a divergence cut-off for this project.

Spatial Assessment Within the Sugarcane Cultivation Region

Imperata cylindrica, *S. arundinaceum,* and *M. capense* showed the highest prevalence within sugarcane cultivation areas (**Table 6**). Three species from Sorghinae, namely *C. sorghoides*, *S. nudipes,* and *Sorghum* ×*drummondii* showed low prevalence within sugarcane QDS (**Table 6**). The highest spatial overlap of wild relatives with QDS containing sugarcane plantations revealed a similar outcome to the prevalence rankings (**Table 7**). In both cases, i.e., prevalence and spatial overlap, the highest and lowest score values differed substantially. *I. cylindrica* showed the highest likelihood for spatial congruence with sugarcane and *S. nudipes* the least.

No collections or observations were made of five wild relatives within sugarcane fields within 700 m of the field margin (**Table 8**). These species can therefore not be considered as common weeds of sugarcane plantations besides the prevalence and spatial overlap with some sugarcane QDS. In general, members of *Sorghum* scored higher rankings for proximity to sugarcane plantations, except for *Sarga versicolor* (**Table 8**), and this is ascribed to preferences for habitat associated with

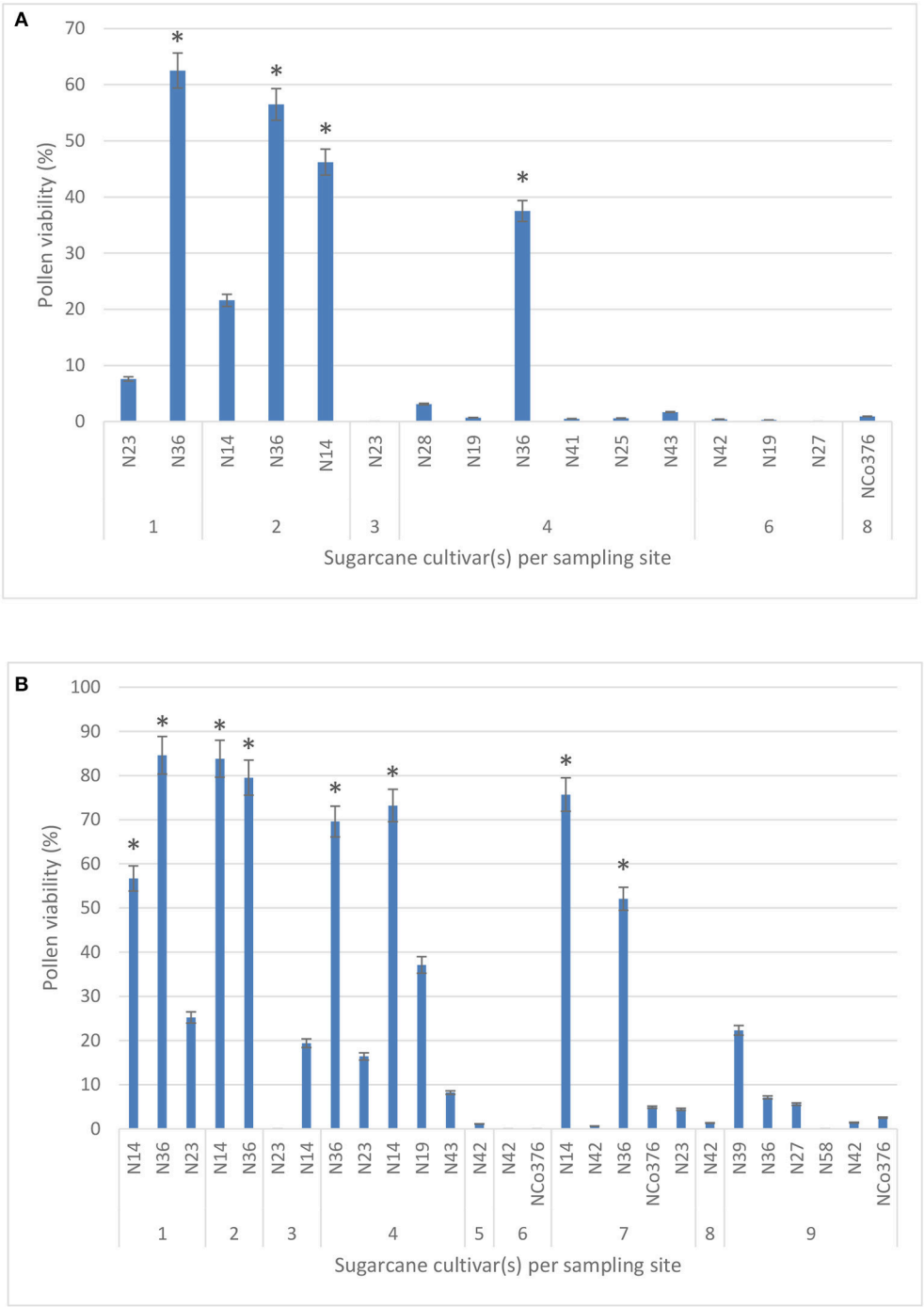

FIGURE 2 | Pollen viability (%) of 11 sugarcane cultivars measured across six study sites using the 2,3,5-triphenyl tetrazolium chloride stain in 2016 **(A)** and 2017 **(B)**. Sites −1: Malelane; 2: Komatipoort (Mpumalanga); 3: Pongola; 4: Jozini; 5: Mtubatuba; 6: Empangeni; 7: Umhlali; 8: Mount Edgecombe; and 9: Port Shepstone (KwaZulu-Natal). Values are means ± SE ($n = 3$). Significant differences are indicated by a * (ANOVA).

sugarcane fields. *Imperata cylindrica* also ranked high, indicating its ability to colozise sugarcane fields. *Miscanthidium* species were moderately associated with sugarcane fields (**Table 8**). Both *I. cylindrica* and *M. capense* were found to be weeds in sugarcane plantations during field surveys although these species were not documented in South African literature as such.

Imperata cylindrica, *M. junceum,* and *S. arundinaceum* were ranked highest in terms of having extensive road and railway networks associated with their QDS of occurrence

TABLE 5 | Flowering times of *Saccharum* wild relatives (based on literature, herbarium specimens, and field observations) in sugarcane cultivation areas.

Species	Flowering period (literature)	Flowering period (herbarium records and field observations)	Combined flowering period	No. months with flowering synchrony	Overlapping months (%)	Score
Cleistachne sorghoides	Feb–Apr	Mar–Apr	Feb–Apr	2	33	2
Imperata cylindrica	Aug–Jun	Jan–Dec	Jan–Dec	6	100	11
Microstegium nudum	Jan–May	Jan–Jun	Jan–Jun	4	67	7
Miscanthidium capense	Nov–Apr	Dec–Jul, Sep	Sep–Jul	5	83	9
Miscanthidium junceum	Nov–Jun	Nov–Jun, Sep	Sep–Jun	4	67	7
Sarga versicolor	Dec–May	Jan–May	Dec–May	3	50	3
Sorghastrum nudipes	Jan–Apr	Jan–Feb, Apr	Jan–Apr	2	33	2
Sorghastrum stipoides	Dec–Apr	Nov–May, Aug	Aug–May	4	67	7
Sorghum arundinaceum	Jan–Jun	Jan–Dec	Jan–Dec	6	100	11
Sorghum ×drummondii	Jan–Jun	Jan–Mar, Jun–Jul, Nov	Nov–Jul	5	83	9
Sorghum halepense	Dec–May	Nov–Mar, May, Jul–Sep	Nov–Sep	4	67	7

Calculation of scores was based on ranking the percentage flowering synchrony with Saccharum hybrids (flowering from March to August in South Africa). Saccharum wild relative species were ranked from highest to lowest, with highest overlap scoring 11 and lowest 1.

(**Table 9**). These networks present a higher likelihood for these species to spread into and within sugar cultivation areas compared with species that have fewer distribution networks. Species that are in isolated QDS and that are normally restricted to certain locations will also lack these distribution networks.

Gene Flow Likelihood

Imperata cylindrica scored the highest during the spatial and temporal assessment, followed by *S. arundinaceum* and *M. capense* (**Table 10**). *M. junceum*, *Sorghum ×drummondii,* and *S. halepense* are further species with high scores. However, based on the relatedness assessment, *I. cylindrica* and the above *Sorghum* species are not closely related with commercial sugarcane (**Figure 2**) and are therefore not candidates to consider for gene flow. A likelihood score based on spatial, temporal and relatedness assessments (**Figure 5**) highlighted the two *Miscanthidium* species. Although *S. arundinaceum* had the highest overall score its distance from *Saccharum* in the phylogeny generated in our study makes it low risk for out crossing. Species with low scores are not considered to present any likelihood for gene flow, especially if these species have diverged from *Saccharum* at more than 7.3 million years (e.g., *Sorghum*).

Closely related species with high spatial congruity pose the highest likelihood for gene flow and certain areas can be flagged where this is the case. No sugarcane QDS with very high likelihood for gene flow was found in Limpopo but there were two of high likelihood in Modjadjiskloof and Tzaneen (**Figure 5**). There was one QDS with very high likelihood in Nelspruit in addition to one QDS with high likelihood in Mpumalanga province. Thirteen QDS with high and 7 with very high likelihood were identified for KwaZulu-Natal, namely Durban, Felixton, Gingindlovu, Port Edward, Port Shepstone, Richards Bay, and Verulam. Overall it appears as if coastal and southern-inland KwaZulu-Natal have the highest likelihood for gene flow to occur based on relatedness, temporal and spatial congruity (**Figure 5**).

DISCUSSION

Several studies have assessed the potential hybridization between plants and their closest relatives in GM scenarios (Ellstrand et al., 1999; FitzJohn et al., 2007; McGeoch et al., 2009) and similar evaluations have been made in sugarcane (Bonnett et al., 2008; Cheavegatti-Gianotto et al., 2011; Organisation for Economic Cooperation and Development, 2013). Our study was designed to consider these factors in a South African context. A review by Ellstrand et al. (1999) listed sugarcane amongst the world's important crop species which hybridize with wild relatives in agricultural systems. Commercial sugarcane cultivars have not been reported to spontaneously hybridize with any related genera and in the two published reviews that assessed the likelihood of GM sugarcane outcrossing with wild species there was no evidence of natural hybridization (Bonnett et al., 2008; Cheavegatti-Gianotto et al., 2011).

Imperata, Sorghum, Narenga, and *Zea* are genera found in South Africa that have been artificially crossed with sugarcane, and evidence of introgression has been confirmed on a molecular level (except in *Imperata*) (Bonnett et al., 2008; Hodnett et al., 2010). It was evident that sugarcane has a considerably low success of producing hybrids compared with its progenitors (i.e., *Saccharum officinarum*) (Piperidis et al., 2000; Aitken et al., 2007). Cheavegatti-Gianotto et al. (2011) noted that even when the barriers to hybridization were eliminated in artificial crosses (i.e., where flowering was synchronized, male pollen viability was increased and numerous florets were hand pollinated), there was poor growth and low survival in seedlings of the progeny. Even though *Saccharum* has previously crossed with *Sorghum* and *Miscanthidium* (Bourne, 1935; Brett, 1954; Gupta et al., 1978), Bonnett et al. (2008) concluded that these genera are unlikely to interbreed either spontaneously or without intervention from breeders due to the low survival rate of the seedlings.

Although the spatial assessment, both prevalence and spatial overlap, confirmed that *I. cylindrica*, *S. arundinaceum,* and *M. capense* had the highest spatial congruence within sugarcane

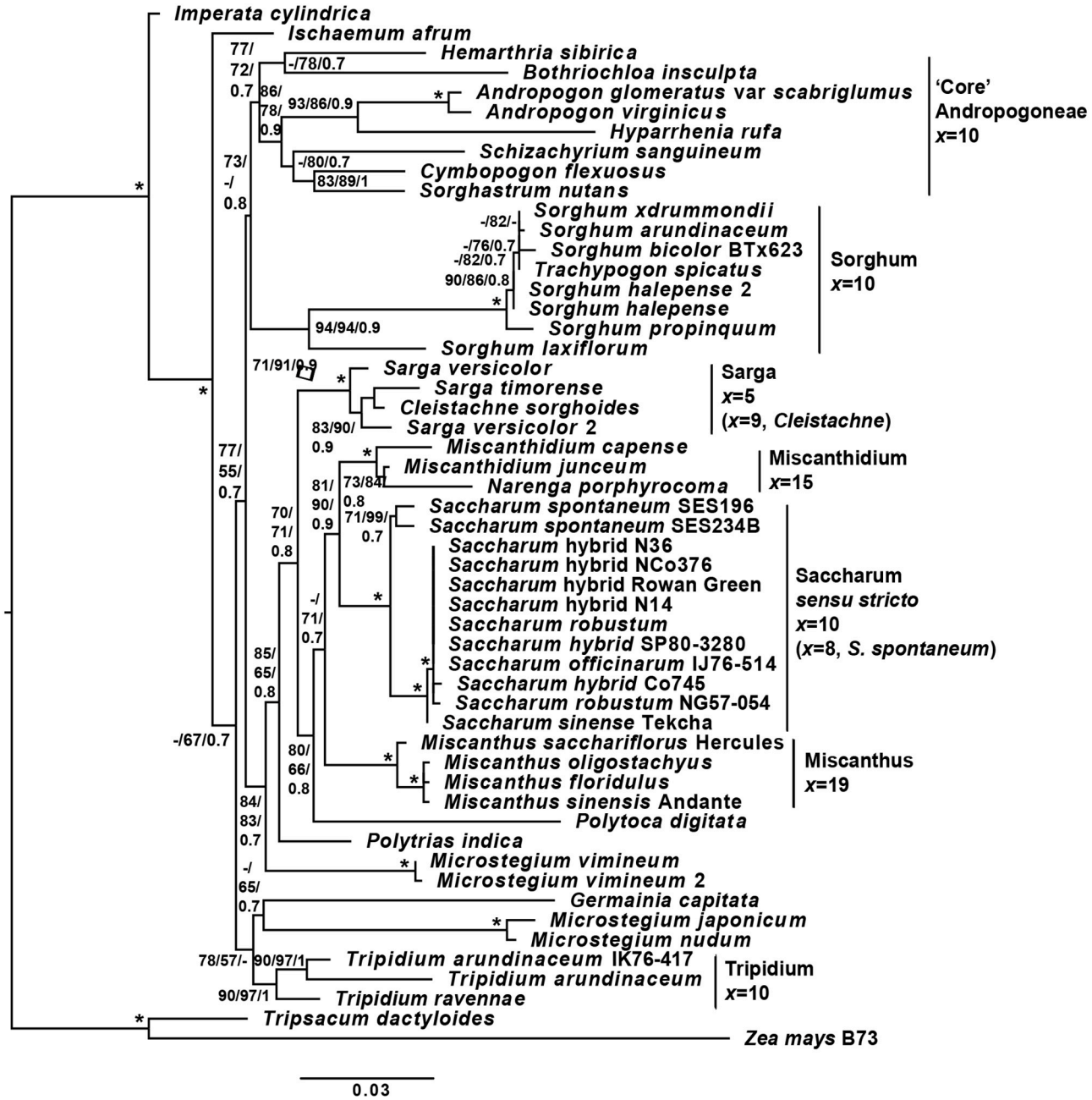

FIGURE 3 | Phylogeny of sugarcane and related genera, based on the ITS cassette. A phylogeny of *Saccharum*, *Sorghum* and related genera based on the ITS (18s rRNA partial, ITS1 complete, 5.8s rRNA complete, ITS2 complete and 28s rRNA partial) genomic cassette. Tree terminals are the species name and cultivar or accession, where appropriate. Numbers at nodes represent SH-aLRT/non-parametric bootstrap/Bayesian inference support values. Bars to the right of the tree represent major clades, with associated base or monoploid (*x*) chromosome numbers. Branch lengths (scale on the bottom) correspond to the expected numbers of substitutions per sides. Monoploid chromosome numbers are derived from: *Sorghum* and *Sarga*—Gu et al. (1984); *Miscanthus*—Adati (1958); *Miscanthidium*—Strydom et al. (2000); *Saccharum spontaneum*—Ha et al. (1999); *Saccharum officinarum*—Li et al. (1959); *Tripidium*—Jagathesan and Devi (1969); and *Cleistachne*—Celarier (1958). The code *represents complete support for a node (100% SH-aLRT, 100% non-parametric boostrap and Bayesian inference of 1), whilst—represents support that is below the threshold (65% for SH-aLRT, 50% for non-parametric bootstrap and 0.7 for Bayesian inference). Within *Saccharum sensu stricto*, between the sister relationship of *Saccharum robustum* NG57-054, *Saccharum* hybrid cv Co745 and *Saccharum officinarum* IJ76-514 with the remaining species there was insufficient sequence divergence within the ITS cassette to yield any meaningful branch supports between the species. The Tripsacinae (*Tripsacum dactyoides* and *Zea mays*) were employed as an outgroup.

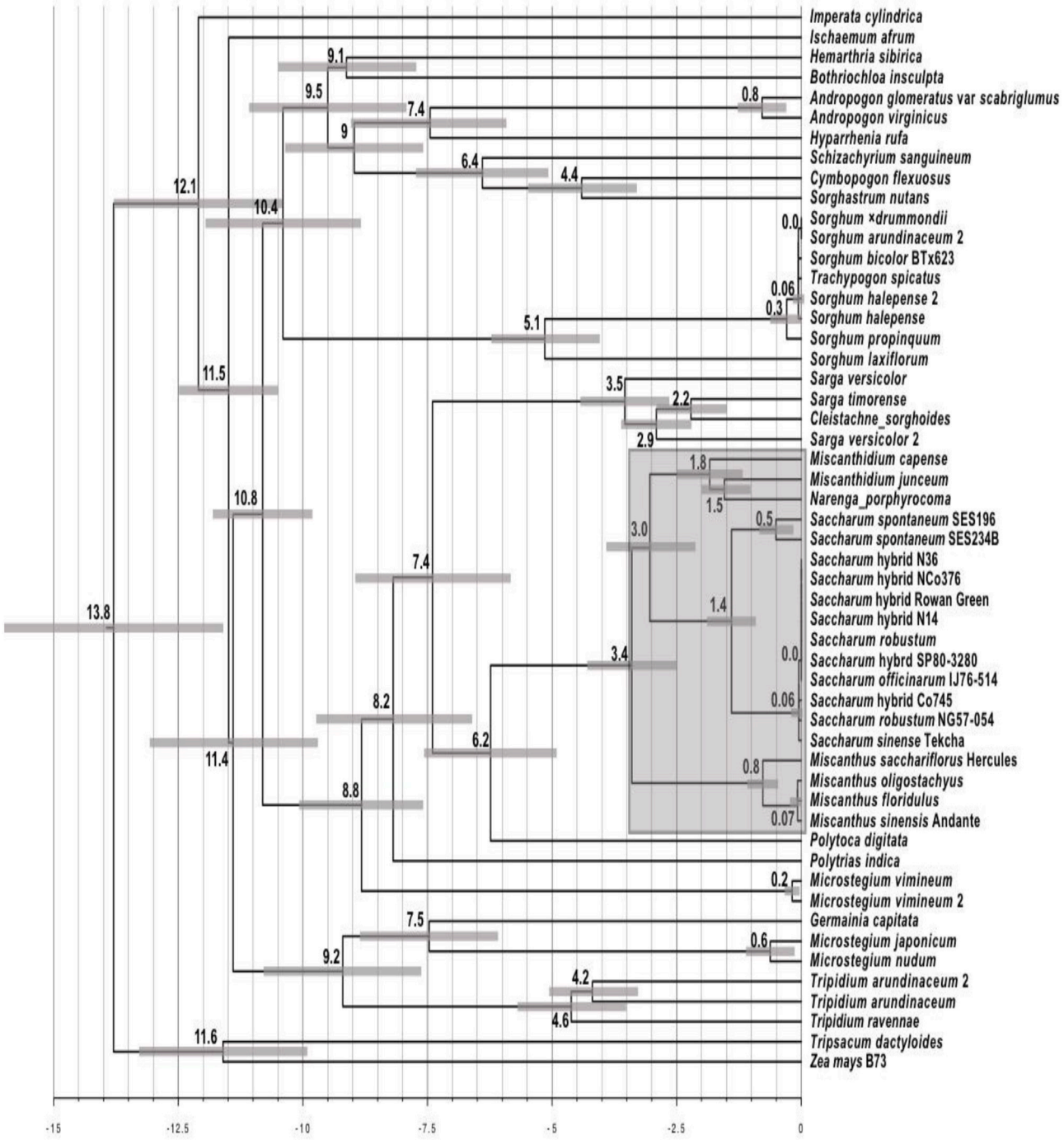

FIGURE 4 | Chronogram derived from the alignment of Andropogoneae ITS cassette sequences. The chronogram was generated with r8s from the Maximum Likelihood ITS phylogeny from **Figure 3**. The scale at the bottom represents millions of years before present. Numbers at nodes represent the age of that node as millions of years before present. Scale bars at nodes represent the central 95% of the age distribution (i.e., 95% confidence interval) as determined by bootstrap resampling. The shaded region centered on *Saccharum* represents the 3.4 million year window in which wild hybridizations between *Saccharum* and other genera is possible.

cultivation areas (**Tables 4**, **6–8**), and synchronous flowering times could facilitate gene flow (**Table 5**), evidence gathered in the present study using phylogenetic analyses of the ITS cassette demonstrated that commercial sugarcane cultivars were sister to *Miscanthidium* species and *Narenga*, but were only distantly related to *S. arundinaceum* and *I. cylindrica* (**Figure 3**).

TABLE 6 | Prevalence or commonness of individuals (based on herbarium specimens) of *Saccharum* wild relatives in sugarcane cultivation areas.

Species	No. individuals within sugarcane QDS	Proportion of individuals within sugarcane QDS	No. individuals bordering sugarcane QDS	Proportion of individuals bordering sugarcane QDS	Total proportion of sugarcane QDS + bordering sugarcane QDS	Score
Cleistachne sorghoides	2	1	3	2	3	3
Imperata cylindrica	99	33	31	25	58	11
Microstegium nudum	10	3	14	11	14	5
Miscanthidium capense	35	12	14	11	23	9
Miscanthidium junceum	15	5	13	11	16	7
Sarga versicolor	10	3	20	16	19	8
Sorghastrum nudipes	1	0.3	1	1	1.3	1
Sorghastrum stipoides	26	9	8	7	16	7
Sorghum arundinaceum	85	28	14	11	39	10
Sorghum ×drummondii	3	1	1	1	2	2
Sorghum halepense	16	5	4	3	8	4

Calculation of scores was based on ranking the commonness of species from highest to lowest, with most common species scoring 11 and least common receiving 1.

TABLE 7 | Spatial overlap (shared occurrence) of *Saccharum* wild relatives (based on herbarium specimens) with sugarcane cultivation areas (113 QDS).

Species	Sugarcane QDS	Overlapping %	Score
Cleistachne sorghoides	2	2	2
Imperata cylindrica	38	34	11
Microstegium nudum	5	4	4
Miscanthidium capense	19	17	9
Miscanthidium junceum	10	9	5
Sarga versicolor	15	13	7
Sorghastrum nudipes	1	1	1
Sorghastrum stipoides	14	12	6
Sorghum arundinaceum	35	31	10
Sorghum ×drummondii	3	3	3
Sorghum halepense	16	14	8

Calculation of scores was based on ranking species occurrences from highest to lowest, with highest ranked species being scored 11 and lowest scoring 1.

TABLE 8 | Proximity or closeness of *Saccharum* wild relatives (based on herbarium specimens, field observations and literature) to sugarcane fields in cultivation areas.

Species	Recorded from field and margins (fm)	Literature confirmations (li)	fm + li	Score
Cleistachne sorghoides	–	–	Absent	0
Imperata cylindrica	7	1	8	10
Microstegium nudum	–	–	Absent	0
Miscanthidium capense	3	–	3	7
Miscanthidium junceum	1	–	1	6
Sarga versicolor	–	–	Absent	0
Sorghastrum nudipes	–	–	Absent	0
Sorghastrum stipoides	–	–	Absent	0
Sorghum arundinaceum	25	2	27	11
Sorghum ×drummondii	3	1	4	9
Sorghum halepense	3	1	4	9

Calculation of scores was based on ranking species proximity to fields from highest to lowest, with highest ranked species being scored 11. A score of 0 was given when no records could be found and therefore proximity data is not currently known (absence equates to no ranking).

It is generally accepted (Kellogg, 2013) that the "core" Andropogoneae (**Figure 3**) defines the dividing line between species that could be part of the Saccharinae and those that are not. Our phylogeny (**Figure 3**) clearly places *I. cylindrica* and *Ischaemum afrum* outside the Saccharinae. The same is true for genus *Tripidium* (Asiatic species). We also place *Sorghum* as sister to the core Andropogoneae (as has also been reported by Hawkins et al., 2015). This means that *Sorghum* is over 11 million years distant from *Saccharum*; well outside the natural hybridization window. *Polytrias indica* and *M. vimineum* form outgroups to the core Saccharinae. *Sarga* is sister to the core Saccharinae, but this is essentially an Asiatic genus; the one exception being *C. sorghoides*, which is native to Eastern Africa from Mpumalanga to Ethiopia (Clayton et al., 2006). However, with a base chromosomal number of 9 (Celarier, 1958), *Cleistachne* is unlikely to be karyotypically compatible with sugarcane.

Miscanthus and *Polytoca*, which are sister to *Saccharum* are Asiatic species as well. The next grouping, which is directly sister to *Saccharum sensu stricto* includes the African *Miscanthidium* species as well as *Narenga porphyrocoma*, which is mainly Asiatic, but has a rump population in Ethiopia. In an African context, at least in terms of evolutionary distance, these are the species most likely to hybridize with *Saccharum*. *Narenga–Saccharum* hybrids have been generated in breeding programmes, but they tend to be male sterile and suffer chromosomal loss in the F2 generation (Price, 1957). Chloroplast data (D Lloyd Evans, personal communication) indicates that *Narenga* hybridized with

TABLE 9 | Distribution potential of *Saccharum* wild relatives (based on road and railway networks) in sugarcane cultivation areas.

Species	QDS with railway line (rl_1)	QDS with railway line bordering (rl_2)	QDS with roads (rd_1)	QDS with roads bordering (rd_2)	$rl_1 + rl_2 + rd_1 + rd_2$	Rank
Cleistachne sorghoides	6	14	7	28	55	1
Imperata cylindrica	49	65	85	165	364	11
Microstegium nudum	7	29	11	55	102	5
Miscanthidium capense	25	38	41	77	181	7
Miscanthidium junceum	36	63	59	161	319	10
Sarga versicolor	18	29	37	101	185	8
Sorghastrum nudipes	5	8	8	44	65	2
Sorghastrum stipoides	12	26	18	37	93	4
Sorghum arundinaceum	28	57	60	164	309	9
Sorghum ×drummondii	4	20	6	42	72	3
Sorghum halepense	16	36	21	85	158	6

Calculation of scores was based on ranking species from highest to lowest using the number of roads and railways present in the grids of wild relatives, and scoring the largest network as 11 and the smallest 1.

TABLE 10 | Score per species calculated by equal weighting of factors obtained for each of spatial (prevalence, spatial overlap, proximity, and distribution potential), temporal (flowering time), and relatedness [hybridization and phylogenetics (**Figure 3**)] assessments.

Species	Prevalence	Spatial overlap	Proximity	Distribution potential	Spatial assessment	Temporal assessment	Hybridization	Phylogenetics	Relatedness assessment	Likelihood score S:T:R (1:1:2)
Sorghum arundinaceum	10	10	11	9	10	11	11	6	8.5	38
Miscanthidium capense	9	9	7	7	8	9	8	11	9.5	36
Miscanthidium junceum	7	5	6	10	7	7	8	11	9.5	33
Sorghum ×drummondii	2	3	9	3	4	9	11	6	8.5	30
Imperata cylindrica	11	11	10	11	11	11	7	1	4	30
Sorghum halepense	4	8	9	6	7	7	9	6	7.5	29
Microstegium nudum	5	4	0	5	4	7	0	7	3.5	18
Sarga versicolor	8	7	0	8	6	3	0	9	4.5	18
Sorghastrum stipoides	7	6	0	4	4	7	0	3	1.5	14
Cleistachne sorghoides	3	2	0	1	2	2	0	9	4.5	13
Sorghastrum nudipes	1	1	0	2	1	2	0	3	1.5	6

Gene flow likelihood score was calculated by weighting the spatial, temporal, and relatedness assessments at 1:1:2.

Saccharum more recently than *Miscanthidium,* and thus may contain more compatible chromosomes.

Miscanthidium species have a base chromosome number of 15 and show no recent hybridization with sugarcane (the two genera have been isolated for at least 2.5 million years). Thus it is likely that *Miscanthidium* and *Saccharum* are not chromosomally compatible. As an Asiatic and Ethiopian species, *S. narenga* poses no threat to gene flow with South African sugarcane, but could be a bridge species in a broader African context. It should be noted however, that of all the genera presented in the phylogeny (**Figure 3**) only the Asiatic and Polynesian species, *Miscanthus floridulus* has categorically been demonstrated to have hybridized with *Saccharum* in the wild (Lloyd Evans and Joshi, 2016a).

As sugarcane hybrids are based on a small number of inter-related parental lines, it is hardly surprising that these cultivars could not be resolved in the ITS phylogeny and the ITS cassette itself does not possess sufficient characters to resolve recently diverged species or cultivars. However, we see that the two *S. spontaneum* accessions are clearly divergent from the other *Saccharum* species or cultivars. *S. sinense* cv Tekcha emerges

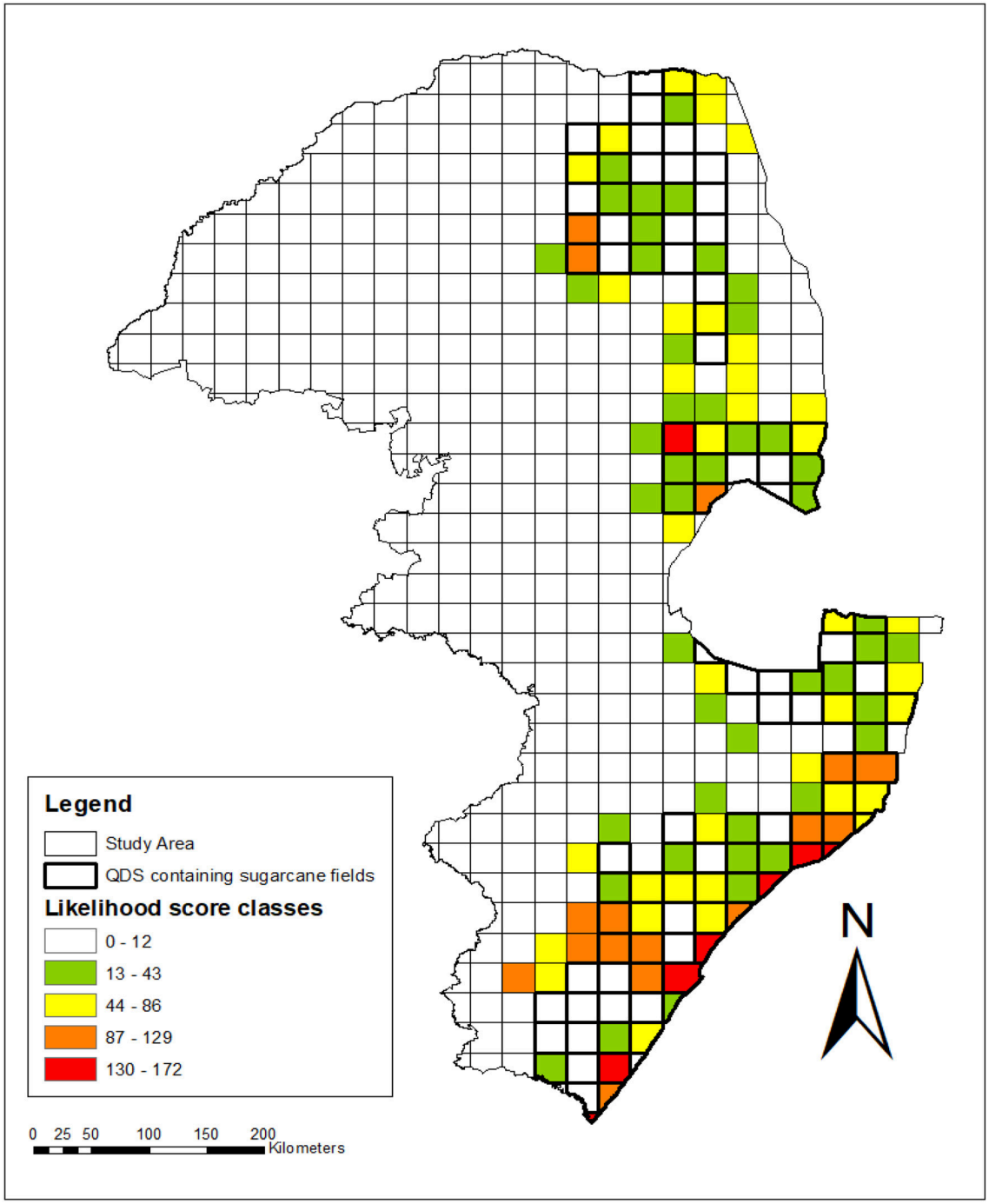

FIGURE 5 | Spatial, temporal and relatedness assessment indicating the levels of likelihood for gene flow to occur between sugarcane and wild relatives in the sugar production region of South Africa. Grid values were calculated by summing the likelihood scores allocated per species (from **Table 10**) for all the species recorded per grid. QDS with sugarcane plantations are indicated with bold lines, whereas other QDS of the study area without sugarcane plantations are not shown with bold lines. Likelihood for gene flow: *Sorghastrum nudipes* scored 6 and there was no sugarcane QDS containing only this wild relative species. QDS with sugarcane plantations without wild relatives (0–12); sugarcane QDS plantations with wild relatives: very low (13–43); low (44-86); high (87–129); very high (130–172).

as ancestral to the remaining *Saccharum* species with 100% support. This is not unexpected as *S. sinense* accessions are ancient hybrids of *S. officinarum* and *S. spontaneum* (Irvine, 1999). As a grouping, *S. robustum* NG57-054, *Saccharum* hybrid cv Co745 and *S. officinarum* IJ76-514 were also resolved from the sugarcane hybrids with 100% support, though resolution within the monophyletic grouping was not possible.

The chronogram (**Figure 4**) provides timings for the radiation events undergone by species analyzed in this study. Few genera lie within the 3.4 million year window where wild hybridization is possible between *Saccharum* and other genera. Even if this window is extended to 7.4 million years, this only adds an additional two genera. All members of *Sorghum* (including *Trachypogon spicatus*) can be excluded as they are 10.4 million years divergent from *Saccharum*. The same applies to *I. cylindrica*, which is 12.1 million years divergent. Interestingly, the chronogram places *Tripidium* species (which sugarcane breeders have been attempting to introgress into *Saccharum* hybrid cultivars for over 50 years with poor success) as 11.4 million years divergent from *Saccharum*. The Southern African species, *C. sorghoides* lies within the genus *Sarga* which is 7.4 million years divergent from sugarcane. However, this species poses low risk of hybridization as it lies outside the wild hybridization window. The only species of high concern in terms of divergence times from *Saccharum* are those within the genus *Miscanthidium*, most especially *M. capense,* and *M. junceum* which are estimated to be approximately 3 million years divergent from *Saccharum* (**Figure 4**).

An unexpected finding was that commercial sugarcane cultivars N36 and N14 had pollen viability of up to 80% in some regions of South Africa (**Figure 2**). Even though no similar studies conducted field assessments across the sugarcane cultivation regions in South Africa, sugarcane seldom produces viable pollen under natural conditions at Mount Edgecombe (site 8) (Brett, 1950; Horsley and Zhou, 2013). Pollen viability gradually decreased from the northern inland (85%) to the south coastal regions (0%) of the study. Within certain study sites (e.g., site 5), some cultivars showed pollen viability of 70%, while others had <10%. A similar study in Brazil reported 100% viable pollen in some cultivars while others showed pollen viability of <9%, under the same environmental conditions (Melloni et al., 2015). Pollen viability has also been closely associated with genotype (Nair, 1975; Pagliarini, 2000; Melloni et al., 2015).

There is a higher likelihood for gene flow when potential pollen recipients flower at the same time as donor crop species when they are in close proximity (Ellstrand et al., 1999; Chapman and Burke, 2006; Schmidt and Bothma, 2006; FitzJohn et al., 2007; Bonnett et al., 2008; Tesso et al., 2008; Nieh et al., 2014). In the current study, there is only one related species with flower synchrony and shared habitat, *M. capense*, which presents the highest potential for gene flow (**Table 10** and **Figure 5**). Although, as discussed previously, all verified hybrids between sugarcane and numerous species within the Andropogoneae have been created through human mediation. Moreover, in all cases hybrids are typically male sterile (Bremer, 1961; Kandasami, 1961; Aitken et al., 2007; Sobhakumari and Nair, 2014) and in F2 and subsequent generations there is considerable chromosomal loss. Thus no sugarcane hybrid reported thus far is a true hybrid, they are always intergeneric (partial) hybrids. Primarily this is due to chromosome number incompatibility (**Figures 3**, **4**) and reflects the divergent evolutionary history of the major lineages within the Andropogoneae. Whilst there are reports of possible hybridizations between *Saccharum* species and related species in the wild, there have been no reports of wild hybridizations with modern hybrid sugarcane cultivars (Cheavegatti-Gianotto et al., 2011). Again this is an issue of chromosomal compatibility. Wild type *Saccharum officinarum* has a base chromosome count of 60 or 80 (typically the latter), but modern hybrids have a chromosome count of about 136 chromosomes—this is variable in different hybrids, but there are typically 10% *S. spontaneum* chromosomes and 90% *S. officinarum* chromosomes (Bremer, 1961). As a consequence, chromosomal incompatibility is far more likely between modern commercial sugarcane hybrids and wild species than between sugarcane's ancestors and wild species. Indeed, even back crosses of commercial hybrids with their immediate ancestors (*S. spontaneum* and *S. officinarum*) often lead to problems of male sterility (Babu, 1990). For crosses between sugarcane hybrid and wild species of low ploidy, not only is there an issue of chromosome incompatibility due to evolutionary distance, there is the added problem of lack of meiotic pairing due to differential chromosome numbers.

In our study, *I. cylindrica*, *M. capense*, *M. junceum*, *S. arundinaceum*, *S.* × *drummondii,* and *S. halepense* were found in relatively close proximity to sugarcane fields (Supplementary Figure 1). The latest review of invasive grasses of South Africa (Visser et al., 2017) reported *Sorghum* ×*drummondii* and *S. halepense* amongst 256 weedy grasses that were introduced to agricultural systems. Weedy relatives may be considered as higher risk for gene flow potential when they are geographically associated with GM crops (Bonnett et al., 2008; Organisation for Economic Cooperation and Development, 2013). In general, most problematic weeds of sugarcane are in the Andropogoneae (Cheavegatti-Gianotto et al., 2011; Organisation for Economic Cooperation and Development, 2013). *Imperata cylindrica* and members of *Sorghum* have been documented as aggressive weeds of agricultural fields including sugarcane plantations in many countries (Van Oudtshoorn, 1999; Firehun and Tamado, 2006; Bonnett et al., 2008; Organisation for Economic Cooperation and Development, 2013; Takim et al., 2014). *Sorghum arundinaceum* and *S.* × *drummondii* are considered as weeds of sugarcane in South Africa (Van Oudtshoorn, 1999; Milton, 2004; Fish et al., 2015). Studies from Nigeria reported *I. cylindrica* amongst problem weeds of sugarcane (Takim et al., 2014), and both *S. arundinaceum* and *S.* × *drummondii* are regarded as major weeds of sugarcane in Ethiopia (Firehun and Tamado, 2006). For South African situations assessed in this study, although *M. capense* and *M. junceus* may be considered to be weeds in sugarcane fields, they are not considered to be "weedy"[7].

Vehicles are amongst the main factors associated with the spread of weedy grasses in South Africa (Milton, 2004). The transport network therefore gives an indication of the potential for weedy relatives of sugarcane to spread, with denser networks implying higher chances for migrations. Furthermore, sugarcane relatives are often associated with roadsides as a preferred habitat (Retief and Herman, 1997; Van Oudtshoorn, 1999; Fish et al., 2015). Potential distribution networks of related species in our study show that most would be able to spread from the areas in which they are found, for example, *M. capense* is associated with vast road and rail networks (**Table 9**), which suggests that

[7]http://www.hear.org/gcw

anthropogenic activities can enhance seed dispersal and increase gene flow potential (Andow and Zwahlen, 2006) in weedy species.

CONCLUSIONS

Phylogenetic analyses of the ITS cassette showed that the closest wild relative species to commercial sugarcane were *M. capense, M. junceum,* and *N. porphyrocoma*. *Sorghum* was found to be more distantly related to *Saccharum* than previously described. Similarly, *Imperata* is so distant from *Saccharum* that it poses no risk of gene flow. In the wild, no hybrids between modern sugarcane hybrid cultivars and any species have been reported. All documented wild hybrids are between sugarcane's ancestors (*S. officinarum, S. robustum,* and *S. spontaneum*) and a small number of closely related species. The phytogeography assessment indicated that the only wild relatives likely to be recipients of gene flow in the study area are *Miscanthidium* species—*M. capense* was observed to be a weed in cultivated sugarcane plantations but it does not have characteristics that make it "weedy." Consequently, even although some commercial sugarcane cultivars do produce fertile pollen—especially in northern irrigated areas of KZN, there is a low likelihood of hybrids occurring in the natural environment. Therefore in a future scenario where GM sugarcane is cultivated in South Africa, the risk of gene flow to wild relatives is low.

AUTHOR CONTRIBUTIONS

SaS conceived the idea and acquired funding for this research. SB and StS designed the experiments. HK and DK conducted the experimental work, analyzed data and interpreted results for their MSc degrees at North-West University, South Africa. Supervision was provided by SB and StS and co-supervision by JvdB, DC, and SaS. SaS co-ordinated the paper writing and submission. DLE performed assemblies from short read data, generated the alignments, performed and interpreted the phylogenies. All authors reviewed, revised, and approved the final version of 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.2018.00072/full#supplementary-material

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Novel Features and Considerations for ERA and Regulation of Crops Produced by Genome Editing

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Genome editing describes a variety of molecular biology applications enabling targeted and precise alterations of the genomes of plants, animals and microorganisms. These rapidly developing techniques are likely to revolutionize the breeding of new crop varieties. Since genome editing can lead to the development of plants that could also have come into existence naturally or by conventional breeding techniques, there are strong arguments that these cases should not be classified as genetically modified organisms (GMOs) and be regulated no differently from conventionally bred crops. If a specific regulation would be regarded necessary, the application of genome editing for crop development may challenge risk assessment and post-market monitoring. In the session "Plant genome editing—any novel features to consider for ERA and regulation?" held at the 14th ISBGMO, scientists from various disciplines as well as regulators, risk assessors and potential users of the new technologies were brought together for a knowledge-based discussion to identify knowledge gaps and analyze scenarios for the introduction of genome-edited crops into the environment. It was aimed to enable an open exchange forum on the regulatory approaches, ethical aspects and decision-making considerations.

Keywords: genome editing, environmental risk assessment (ERA), regulation, new breeding techniques (NBT), CRISPR/Cas, ISBR, ISBGMO

INTRODUCTION

New plant breeding techniques, such as genome editing, enable a previously unachievable targeted and precise modification of the genome. They allow for the introduction of very precise genomic changes, ranging from the exchange, insertion or deletion of one nucleotide at one specific locus to the site-specific integration of entire genes. Genome editing comprises protein mediated techniques (e.g., TALENs, zinc-finger nucleases), nucleic-acid-mediated genome modifications (e.g., ODM), or a combination thereof (e.g., CRISPR-techniques). Molecularly, in most cases a DNA double strand break (DSB) is induced which is subsequently repaired by one of the endogenous cell

repair mechanisms, non-homologous end joining (NHEJ) or homologous recombination (HR). The preferential repair mechanism in plants is NHEJ, which is prone to errors. Due to these errors small changes in the nucleotide sequence (mutations) can be induced at the repaired locus (Hsu et al., 2014; Bortesi and Fischer, 2015) which may result in variants useful to crop improvement.

On-going development and innovation in genome editing promise to increase its value as a tool for crop improvement. For instance, a modified Cas-nuclease allows the precise editing of target bases in genomic DNA without relying on double-strand breakage (Komor et al., 2016). In multi-target approaches, site-directed mutagenesis of several target genes can be tackled simultaneously (Svitashev et al., 2015; Chilcoat et al., 2017; Shen et al., 2017), and targeted transgene insertions at one specific locus (Ainley et al., 2013) could lead to trait stacking possibilities with yet unknown dimensions. All these developments occurred within just the last few years, and rapid progress is to be expected (Puchta, 2017).

Those genome editing applications that do not aim at the insertion of foreign genes, but at inducing site-specific mutations at single loci of a plant's own genetic material, are able to create organisms that could have come into existence naturally or through conventional breeding. Thus, although few regulators in some countries have instituted mechanisms for addressing the regulatory status of crops derived from genome editing (Whelan and Lema, 2015; Wolt et al., 2016), decisions as to whether or not they require legal regulation lag behind in most countries.

In the EU, it is still unclear whether edited organisms will fall under the Genetic Engineering Law. The court cases on CIBUS™ canola are pending in Germany, and no legal guidance has been published by the European Commission (for a summary see Sprink et al., 2016). Legal classifications of new plant breeding techniques, including various genome editing tools, were suggested by an independent EU member states expert team in 2011 (Lusser et al., 2011), but the report by a "New techniques Working Group" set up by the European Commission to assess whether or not plants created by certain breeding techniques fall within the scope of the genetically modified organism (GMO) legislation, which was finalized in 2011, has never been officially released (Kahrmann et al., 2017). The European Commission has not published any legal opinion on these techniques so far and is not expected to do so at short notice. During the hearing of October 3, 2017 in the Case C-528/16 at the European Court of Justice, the Commission vaguely stated that they were preparing something about this "new" problem. In contrast, according to its statement during that hearing, the Commission is of the opinion that mutagenesis is exempted from the Directive on deliberate release if no recombinant nucleic acid molecules are used.

And indeed, with the exception of Canada, regulatory authorities throughout the world do not consider mutagenesis as subject to regulation under biosafety laws. And even in Canada, traditional mutagenesis is not regulated unless it produces a novel trait. It is the novel trait that is regulated, not the method used to produce it.

In the USA, current decisions on genome-edited plants have been based on the Plant Protection Act, as enforced by the USDA. The US Coordinated Framework for Biotechnology makes no special provisions for genome edited crops. As for any biotechnology-derived plant, if the genome-edited crop poses a plant pest risk, expresses a pesticide trait, or poses food safety risks different from other plants produced through traditional plant breeding then it is subject to regulatory considerations (by USDA, EPA and FDA, respectively); otherwise, the product can freely enter market channels. A new regulatory framework for biotechnology that was drafted in the last 2 years was going to be based on a noxious weed designation, but it was withdrawn in late 2017 to re-engage with Stakeholders (USDA, 2017)[1]. USDA does not currently regulate, or have any plans to regulate plants that could otherwise have been developed through traditional breeding techniques as long as they do not pose a plant pest risk, that is, as long as they are developed without the use of a plant pest as the DNA donor or transformation vector and they are not themselves plant pests or noxious weeds (USDA, 2018)[2]. In addition, both the FDA and the EPA could regulate genome-edited crops, but neither agency has indicated what their approach will be.

Elsewhere, regulatory frameworks have been established that allow for progress in the development and commercial advancement of crops developed through genome editing, even as the specifics of the regulatory frameworks are being considered (Whelan and Lema, 2015; Wolt et al., 2016).

With the current lack of adequate legal guidance throughout much of the world, a debate has started whether the legal status of plants derived from genome editing has to be based on the process used to create the organism (process-based approach) or on the final product obtained by the process (product-based approach). Another point of discussion is whether point mutations created by genome editing techniques have the same legal status as point mutations created by spontaneous or by conventional induced mutagenesis. Moreover, in various countries, traceability requirements are in use to detect and to identify a GMO[3]. Yet, most point mutations—or even larger changes, as long as no foreign DNA is integrated into the final organism's genome—do not carry a tag displaying the technique used to create them. Finally, asynchronous or even contradictory regulation of organisms created by genome editing in different countries will disrupt world trade and collide with standards of the World Trade Organization (WTO).

Rapid progress in genome editing technologies is challenging risk assessment and post-market monitoring frameworks: Shall certain types of genome-edited crops pass a simplified procedure on risk assessment as was suggested by Huang et al. (2016)?

[1]USDA (2017) *Press. Release No. 0144.17*.

[2]USDA (2018) *Details on USDA Plant Breeding Innovations*. https://www.aphis.usda.gov/aphis/ourfocus/biotechnology/brs-news-and-information/pbi-details

[3]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."

Shall the types of genome edited crops that also could have been created by conventional breeding techniques (e.g., by classical mutagenesis) be regulated no differently from conventionally bred crops? Does the current scientific development represent the ultimate trigger to now design a novel framework for risk assessment which focusses on the product and its potential effects on health and environment irrespective of the technique used to develop it, as suggested by Conko et al. (2016)?

To help answer these questions, the session "Plant genome editing—any novel features to consider for ERA and regulation?" held at the 14th ISBGMO[4], used both, expert presentations and an interactive "World Café" discussion to bring together scientists from various disciplines (molecular biology, modeling, genetics, ecology) as well as regulators, risk assessors and potential users of the new technologies. Recent technological developments were summarized and examples of current applications in plant breeding were collected. A science-based discussion was aimed at the identification of knowledge gaps and the analysis of scenarios for the introduction of selected edited organisms into the environment. The interactive session raised awareness of benefits and risks of the new techniques and provided the opportunity for an open exchange, connecting regulatory approaches, ethical aspects and decision-making.

Key Expert Contributions on Challenges, Opportunities and Perspectives of Genome Editing Applications for Crop Plant Breeding

Five talks were given in the session that provided a framework for the following discussion and "World Café." Each talk focused on a different but major aspect or perspective relevant to the question at hand.

Risk assessment often starts by identifying hazards that are unique to the item being regulated. Accordingly, Wayne Parrott compared the result of genome editing with conventional plant breeding in an attempt to identify any new and unique features about genome editing:

The technology for genome editing developed very rapidly, and equally quickly found numerous applications across medicine and agriculture. The latter includes the modification of crop plant genomes. Due to the novelty of the technology, many groups are singling out edited plants as somehow being new and different from plant varieties produced in the past.

To properly address the question about what is unique, or at least new and different, about genome editing, it is first necessary to consider how new plant varieties are produced by conventional plant breeding. The changes that take place at the chromosome level during the breeding process are of particular relevance, as they serve a basis for comparison of the changes made by genome editing.

Modern row crop varieties or cultivars can be thought of as collections of various traits. These traits can affect the phenology of the plant, the quality of the product, or provide agronomically useful traits, such as resistance to abiotic stress, pests, and pathogens. Today, most seed catalogs will list all the relevant phenological traits and all the resistances found in any given cultivar or hybrid.

Each of these traits is the result of one or more genes. Usually, it is a matter of identifying the right allele of the gene, and breeding the desired allele into the cultivar. Aside from a few epigenetically controlled traits, all heritable traits reflect changes that take place at the DNA level (Weber et al., 2012; Schnell et al., 2015). Thus different alleles of a gene have different DNA sequences. The difference in the sequence can be as small as the substitution of a single base pair, or can consist of base pair deletions or insertions that range from single base pairs to thousands of base pairs.

A plant breeder will first search for desired alleles in other varieties of the same crop, or in its landraces or wild relatives (Acquaah, 2012). Failing that, a breeder may try to bring out variation through mutagenesis (Ahloowalia et al., 2004). The changes made by mutagenesis to the DNA range from single base pair substitutions, to inversions, insertions, and deletions of various sizes (Anderson et al., 2016).

Another alternative for breeders is to find the desired trait in a related species. Specialized laboratory procedures may be necessary to make the cross, but the practice has been on-going for the past century (Jones et al., 1995; Hajjar and Hodgkin, 2007). Any variety produced with genes from another species is technically a transgenic; it just does not count as a GMO because recombinant DNA was not used to move the gene from one species to the other.

Finally, the traditional concept in plant breeding has been that all plants have the same genes, and all that breeders have been doing is replacing the allele of one gene with another allele. The on-going, large-scale sequencing of plant genomes revealed that the traditional perspective is not completely correct, and different varieties of a crop differ by the presence and absence of hundreds, if not thousands, of genes (e.g., Agnieska et al., 2016; Hirsch et al., 2016). Thus breeders have not just been replacing alleles, they also have been inadvertently adding whole genes. The relevance is that genomes are clearly not adversely affected by the presence or absence of many genes, nor are there quantifiable safety issues associated with adding or removing genes.

Collectively then, plant genomes are modified by a series of natural and artificial processes that result in the genetic variability used by breeders (**Figure 1**). With this background, it becomes possible to compare the changes at the DNA level that differentiate alleles from each other with those made by genome editing. Although the term "genome editing" implies a single process, editing can have three distinct effects on the plant genome:

1. A gene can be knocked out, i.e., inactivated by adding or deleting DNA, ranging from single base-pair deletions to deletion of the gene altogether.
2. A gene can be converted from one allele to another, by replacing base pairs from allele with base pairs from another allele.

[4]http://isbr.info/ISBGMO14

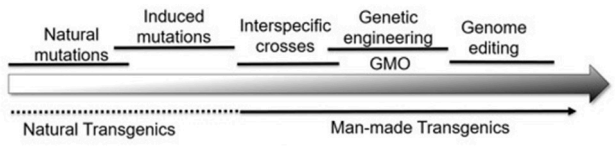

FIGURE 1 | A wide range of natural and artificial processes serves to alter the plant genome in many ways. From a biological perspective, it becomes difficult, if not impossible, to draw vertical lines separating one category of modification from the other.

3. A gene can be inserted in a predetermined place.

Looking at these three changes in more detail:

1. Gene knock-outs—the process creates non-functional alleles. As such there is nothing novel about non-functional alleles, which can be ubiquitous in plant populations. The changes at the DNA level made by editing are indistinguishable from those found naturally, which in turn are like those recapitulated in mutagenesis. In other words, a single-gene knock out from genome editing is indistinguishable from what happens naturally or in mutagenesis. The difference is that editing is more efficient at creating desired knockouts. Since natural and induced mutations take place at random locations, large numbers of plants must normally be screened to find one desired mutant. In contrast, genome editing can be targeted to a specific gene.

   However, few plant genes are found as single genes. Genes frequently are part of gene families. Alternatively, the plant can be an allopolyploid, which means the gene is duplicated on other chromosomes. The only way to get a recessive phenotype is if all gene copies are knocked out, and mutagenesis has never been effective at knocking out genes found in multiple copies (Stadler, 1929). In contrast, genome editing is adept at knocking out genes present in multiple copies. Thus, whenever a crop is found with multiple copies of the same gene knocked out, it will be almost certain that genome editing was used.
2. Converting one gene to another. Such editing recapitulates what breeders routinely do during backcrosses. The key difference is that breeders cannot replace single alleles with another in most species. Instead, they work with blocks of linked genes (Young and Tanksley, 1989). Therefore, genome editing can accomplish the task far more precisely and quickly than conventional breeding can ever do.

   On important difference is that some crop genes lie in low or non-recombinogenic regions of the chromosome. Thus, these genes have not been amenable to backcrossing during plant breeding programs. Genome editing ensures all genes are amenable to allele replacement.
3. Finally, there is site-specific gene insertion, a process that recapitulates the introduction of genes present in one variety but not another during conventional plant breeding. The difference, of course, is that in plant breeding the additional genes come from related species, while the genes can come from any organism when site-directed insertion is used. But then again, all plants are now known to have received genes from unrelated species (e.g., Bock, 2010).

All these considerations inform that genome editing simply creates the types of changes that are commonplace in nature. The main difference is that editing removes much of the randomness out of the process. Since risks always come from the final product and not from the way this product was obtained, there are no identifiable risks associated with editing that are different from those associated with conventional plant breeding, which in turn has a remarkable history of safe use (Steiner et al., 2013). The one exception would be if site directed insertion was to be used to insert a gene that codes for a toxin or an allergen, and procedures to evaluate the safety of novel genes are well established.

A final consideration is that genome editing can have off-target effects—it can create changes in the genome in places other than those intended. To evaluate the consequences of such off-target effects, it is once again necessary to compare genome editing with conventional plant breeding. The single largest source of off-target effects turns out to be conventional plant breeding (NASEM, 2004; Anderson et al., 2016). Likewise, traditional mutagenesis is rife with off-target effects that few people ever bother to detect or characterize. These historically have not been a cause for safety concerns, and the historical safe use of mutagenized crops bears witness to their safety. Thus, while it is possible to optimize the editing process to minimize off-target effects, and that these off-target effects would likely be removed during the subsequent breeding process, there is no reason to believe that any unintended edits left behind would pose a safety concern for crop plants.

In summary then, the unique features of genome editing are (1) its ability to edit genes present in multiple copies and (2) the ability to target the sites in the genome to be edited. At the DNA level, the changes are like those that take place naturally or in mutagenesis and that have a long history of safe use. The inescapable conclusion is that genome editing for gene knockouts and allele replacement must be considered to be at least as safe as conventional breeding. From the perspective of the FDA 1992 policy, edited plants would be subject to the same type of assessment as any traditionally bred variety. In other words, they should not need any special safety assessment.

Thorben Sprink next described the regulatory challenges posed by genome-edited crops from the perspective of a public risk assessor in the EU:

In recent years genome editing and associated techniques have become a frequently used tool not only in research but also in applied breeding. Especially the CRISPR technology was a groundbreaking discovery, which is yet developed further with constantly expanding applications (**Figure 2**). Many traits in plant and animal breeding, as well as for medical application, have been addressed by genome editing and more are in progress (**Figure 3**). But only a handful of these have been subject to regulatory consideration in the US[5] or in Europe (BVL)[6].

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

[6]https://www.bvl.bund.de/DE/06_Gentechnik/04_Fachmeldungen/2015/2015_06_03_Fa_CIBUS.html)

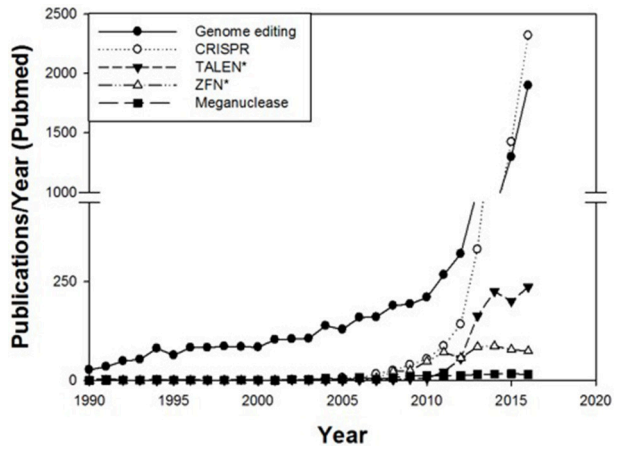

FIGURE 2 | Number of publications per year in PubMed cited for the terms genome editing, CRISPR, TALEN*, ZFN* and meganucleases. The number of publications regarding these techniques has rapidly increased since 2008.

The challenges of a regulatory framework in the face of new emerging technologies is not new to the European Commission. Back in late 2007, a working group was established to analyze a non-exhaustive list of techniques for which it is unclear whether or not they would result in GMO products under the current GMO regulation. The final report, however, has not been published and in the EU no final decisions have been made so far and no legal guidance has been published by the Commission (for a short summary of this topic, see Sprink et al., 2016).

Whether or not there are new environmental risk assessment (ERA) challenges that are associated with genome-edited crops has also been addressed by many scientific organizations. Their opinions and statements have been updated throughout the last 2 years. In 2017, the scientific advice mechanism (SAM) of the EU Commission has been requested to issue "*an explanatory note on new techniques in agricultural biotechnology including their potential agricultural application in synthetic biology and for gene drive, taking into consideration the most recent developments in the agricultural sector*" (SAM, 2016). This report has been published as of April 2017 (SAM, 2017). It compares NBTs with conventional breeding techniques (CBT) as well as with established techniques of genetic modification (ETGM) in seven categories: (i) Detectability/Identification, (ii) Unintended effects, (iii) Presence of foreign DNA, (iv) End product characteristics, (v) Ease of use/efficiency, (vi) Speed and costs and (vii) Maturity.

The SAM report points out that not only NBTs and ETGMs make use of genetic diversity and change to enable a genomic selection, but CBTs do so as well. They conclude that NBTs contain a variety technologies, and that, in some cases, the resulting products are comparable to the products of CBT as they do not contain foreign DNA, while in other cases they are comparable to products of ETGM, as NBTs also enable the use of foreign DNA. The report concludes that NBTs are more precise and result in lower amounts of unintended effects than CBT and ETGM do. Furthermore, especially genome editing techniques show a much lower number or a complete lack of unintended mutations as compared to products obtained via CBT, in particular when compared to mutation breeding or induced mutagenesis. Without prior knowledge of the alterations made to the genome by any of those techniques that do not introduce foreign DNA, the changes will be difficult to detect, and the identification of a particular technique as the cause of a certain alteration is impossible. The SAM declares that a safety assessment can only be made on a case by case basis depending on the traits of the end product or organism (SAM, 2017).

This statement echoes the updated statement of the European Plant Science Organisation (EPSO, 2017) which argued that "the EU regulatory framework for GMOs has become increasingly dysfunctional, as decisions are often not taken within the legal time frames, and often not on the basis of scientific evidence and risk assessment. The requested information and risk assessments are more comprehensive and are galvanized without scientific justification instead of being based on gained knowledge." EPSO additionally calls attention to the point that GMOs should not merely be defined by the use of a certain technique but that a GMO also requires that a novel combination of genetic material beyond the natural borders of mating and recombination has been produced. This is not the case for point mutations obtained by genome editing (EPSO, 2017). Therefore, EPSO is in favor of a process- as well as product-based interpretation of the current framework of the EU and considering this to help to clarify the legal status of the NPBTs. EPSO supports the conclusions of the New Techniques Working Group, "that the legal definition of a GMO does not apply to most NPBTs and that these techniques either fall under the exemptions already established by the legislation or should be exempted as they do not differ from plants obtained by traditional breeding."

The European Academies Science Advisory Council (EASAC) also updated their statement in March 2017 (EASAC, 2017). EASAC concludes that "*policy considerations should focus on the applications rather than on the genome editing procedure itself as an emerging technology. It should be ensured that regulation of applications is evidence based, takes into account likely benefits as well as hypothetical risks, and is proportionate and sufficiently flexible to cope future advances in the science.*" EASAC also focuses on the product as the trigger for regulation by asking EU regulators to "*confirm that the products of genome editing, when they do not contain DNA from an unrelated organism, do not fall within the scope of legislation on genetically modified organisms (GMOs)*". Additionally, EASAC argues for "*a full transparency in disclosing the process used, but that the aim in the EU should be to regulate the specific agricultural trait/product rather than the technologies by which it is produced.*" This implies that the use of new technologies would be exempted from regulation if *"the genetic changes they produce are similar to, or indistinguishable from the product of conventional breeding, and if no novel, product-based risk is identified.*

Users of genome-editing technology for crop improvement face their own set of challenges that frame their perspective. Accordingly, Maria Fedorova gave a product developer perspective on genome editing and its similarities to and advantages over conventional breeding outcomes:

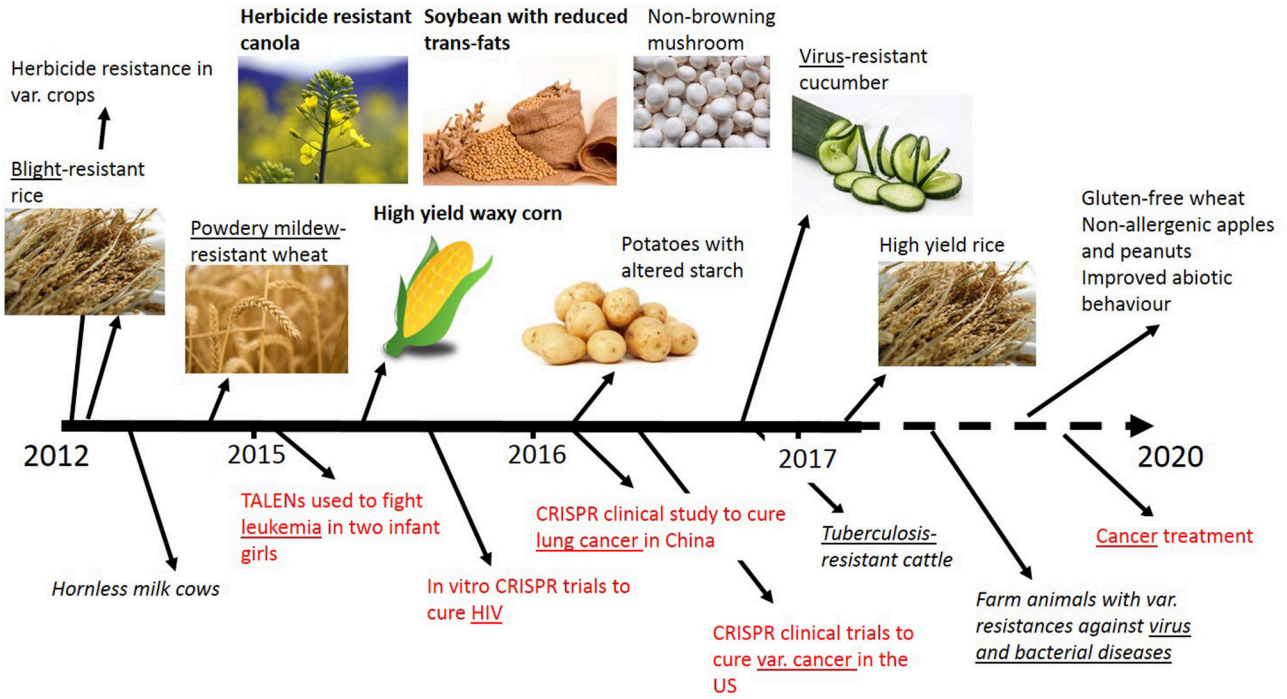

FIGURE 3 | Timeline of selected traits modified by genome editing in plants, animals and for medical applications (red). Addressed diseases are underlined. Bold front: Applied for non-regulated status in the USA. No permissions were required for the use of the images[7].

Traditional plant breeding has historically relied on plant's genetic variability to develop new varieties with improved characteristics. Favorable allelic variations, spontaneous mutations and induced random mutations have been a source of genetic diversity carried forward into commercially valuable genotypes. The ability to induce genetic variation in a targeted and more efficient fashion has been viewed as a much needed breakthrough and a challenge until recently. Genome editing, enabled by tools such as ZFN, TALEN or CRISPR/Cas, provides that breakthrough.

Genome editing can be defined as targeted modification of the plant's own genes without permanently introducing any foreign genetic material. This distinguishes genome-edited varieties from GMOs. Genome editing can produce plants indistinguishable from those that could arise from spontaneous or induced classical mutagenesis or be developed by introgression of the desired allele through a series of breeding crosses—i.e., tools deployed in conventional plant breeding.

CRISPR/Cas is one of the most recent genome editing tools, rapidly expanding its utility for academic research (reverse genetics, functional genomics studies) as well as practical application to develop new crop varieties with improved characteristics. CRISPR/Cas genome editing is viewed as a major advancement in precision plant breeding due to its versatility, efficiency and ability to work across species.

One of the examples of crop improvement using CRISPR/Cas genome editing is the next generation waxy (high amylopectin) maize, which was produced by targeted deletion of the waxy (*Wx1*) gene directly in elite inbred lines (Chilcoat et al., 2017). *Wx*1 is one of the most studied "classical" maize genes, with over 200 various spontaneous or induced mutations (deletions, insertions, translocations of various length) known to lead to the waxy phenotype (Wessler and Varagona, 1985; MaizeGDB, 2017). DuPont Pioneer's conventional waxy maize product, cultivated since the mid-1980s, is based on a spontaneous *Wx1* mutation (sequence deletion in the middle of the gene) from a maize variety discovered over 100 years ago (Fergason, 2001; Fan et al., 2009).

Limitations of the conventional waxy maize products are related to the introgression process of the *Wx1* mutation into top-performing modern elite lines and could be mitigated if the mutation in the *Wx1* gene was accomplished directly in elite inbred lines. Therefore, waxy maize elite inbred lines were generated by targeted *Wx1* mutation using CRISPR/Cas technology (Chilcoat et al., 2017). These lines exhibit the expected waxy phenotype, do not contain plasmid DNA used in the transformation process, and undergo extensive field evaluations according to common breeding practices.

[7]https://pixabay.com/de/reis-und-mais-reis-mais-sorte-korn-587593/; https://pxhere.com/de/photo/707286; https://pixabay.com/de/mais-ernte-gem%C3%BCse-lebensmittel-152037/; https://pixabay.com/de/raps-landwirtschaft-feld-bl%C3%BCte-1333511/; https://pixabay.com/de/sojabohnen-pflanzen-saatgut-tasche-2039639/; https://pixabay.com/de/pilze-champignons-wei%C3%9F-1351060/; https://pixabay.com/de/kartoffeln-erd%C3%A4pfel-ungesch%C3%A4lt-2829118/; https://pixabay.com/de/gurke-salat-lebensmittel-gesund-685704/

It is fully appropriate to consider genome editing in the context of the range of plant breeding methods and, specifically, with the following two perspectives: how different is genome editing from processes occurring in nature or through conventional breeding methods? And, what is the likelihood of any given mutation to create a biosafety risk?

Inherent genetic variability is the biological mechanism allowing plants to adapt to ever-changing internal and external conditions. Genetic diversity is exceedingly common in plants, including important crop species such as maize, soybean, or rice (refer to Ching et al., 2002; Naito et al., 2006; Schnable et al., 2009; Springer et al., 2009; Parrott et al., 2012 for just a few examples). These spontaneously occurring processes are fundamental to crop evolution and the successful development of high performing elite varieties. To increase the genetic diversity, breeders can further boost the mutation rate by deploying classical (chemical, irradiation) mutagenesis tools, which generate multiple additional, random and unknown mutations besides the mutation of interest. As acknowledged by the European Food Safety Authority (EFSA), the frequency of mutations is predicted to be higher after classical mutational breeding (EFSA, 2012). With that, classical mutagenesis is broadly and successfully used in modern plant breeding, with over 3200 mutants registered in the FAO/EAEA mutant variety database[8]. Thus, the history of safe use of conventionally bred varieties demonstrates that a multitude of mutations occurring in a plant's own genes or intergenic sequences is unlikely to impact plant safety. The outstanding track record of conventional plant breeding provides a solid scientific basis for safety comparisons.

CRISPR/Cas genome editing allows to make many types of genetic changes similarly possible through conventional breeding but in a targeted fashion, i.e., in a more efficient, predictable and precise manner. The potential for off-target cutting can be mitigated by a variety of approaches, ranging from robust guide RNA design to modification of experimental conditions and to various molecular diagnostic tools tracing if an off-target cutting has actually occurred (Cameron et al., 2017 and references within; Svitashev et al., 2016). Furthermore, any potential off-target mutation, even if it occurred initially, would have been most likely segregated out during subsequent breeding cycles to develop the commercial variety. The generation of genome-edited plants without off-target mutations has been demonstrated in a number of publications (Baysal et al., 2016; Chandrasekaran et al., 2016; Nekrasov et al., 2017; Sánchez-León et al., 2017, to list a few).

Thus, risk assessment considerations associated with potential unintended effects or off-target cutting in genome-edited plants needs to be viewed in the context of the well-documented dynamic nature and plasticity of plant genomes. Similar to conventionally bred varieties, even if an off-target mutation were to occur, it is not expected to inherently make a genome-edited plant present a greater safety risk than a conventionally bred plant. The potential for unintended changes in the genome is not a unique feature of genome editing where any potential imprecision is expected to be significantly lower than the rates of spontaneous mutations or classical mutagenesis for which there is an established history of safe use.

Regulatory systems may face additional challenges posed by genome-edited products, which were discussed by Martin Lema. These include for instance the debate between technology-based and product-based regulations and the potential impact on product monitoring:

The Core Issue of Regulatory Touchstones

Debates regarding the regulatory status of genome-edited organisms generally follow a comparative approach with GMOs and with conventional organisms obtained by mutation and breeding. In general, these debates began considering *in extenso* technical aspects such as the possibility of generating the same kind of genetic modifications by other means, or the detectability of edited genes for the purpose of control and monitoring, or the relative safety of these products.

Certainly, these aspects are of high relevance. However, in the end regulators have to decide which regulation does or does not apply to a particular product. For this purpose, regulators need to resort to some legal "touchstones," which most often are a definition (such as the GMO definition in most countries), triggers (such as "novel trait" in the Canadian regulatory system) or a list of inclusions/exclusions (like the Australian regulatory system).

Rules that determine whether or not an organism falls under a special GMO regulatory regime differ from one country to another. Quite often, their parameters for regulatory inclusion are based on product characteristics and/or the process used to obtain them. A recent review of the global GMO regulatory landscape which aims at anticipating the future scenario for genome-edited crops shows that the debate on "product-based" vs. "process-based" regulation is not the key influence when it comes to technology adoption (Ishii and Araki, 2017). The article also reports that many national regulations depart from the LMO definition of the Cartagena Protocol.[1] which is worrying since the Protocol should act as a harmonizing factor. But while these diverging definitions have so far not created major issues for the classification of a plant variety as GMO (or comparable categories) or as a conventional crop, genome editing and other NBTs represent a broader spectrum of technical possibilities. The combination of this variety of technical possibilities with the wide array of subtle differences in national regulatory touchstones, may asymmetries that can affect trade.

First Experiences in the Regulation of Genome-Edited Organisms

Debates on the regulatory status of genome-edited organisms (initially under the concept of "new breeding techniques") date back at least to the year 2011 (Lusser et al., 2011). Nevertheless, to date there have been very few official regulatory determinations regarding these products (Wolt et al., 2016; Ishii and Araki, 2017). These articles and references therein provide a complete review of the first regulatory decisions in the USA, Canada, New Zealand and some isolated

[8]https://mvd.iaea.org/

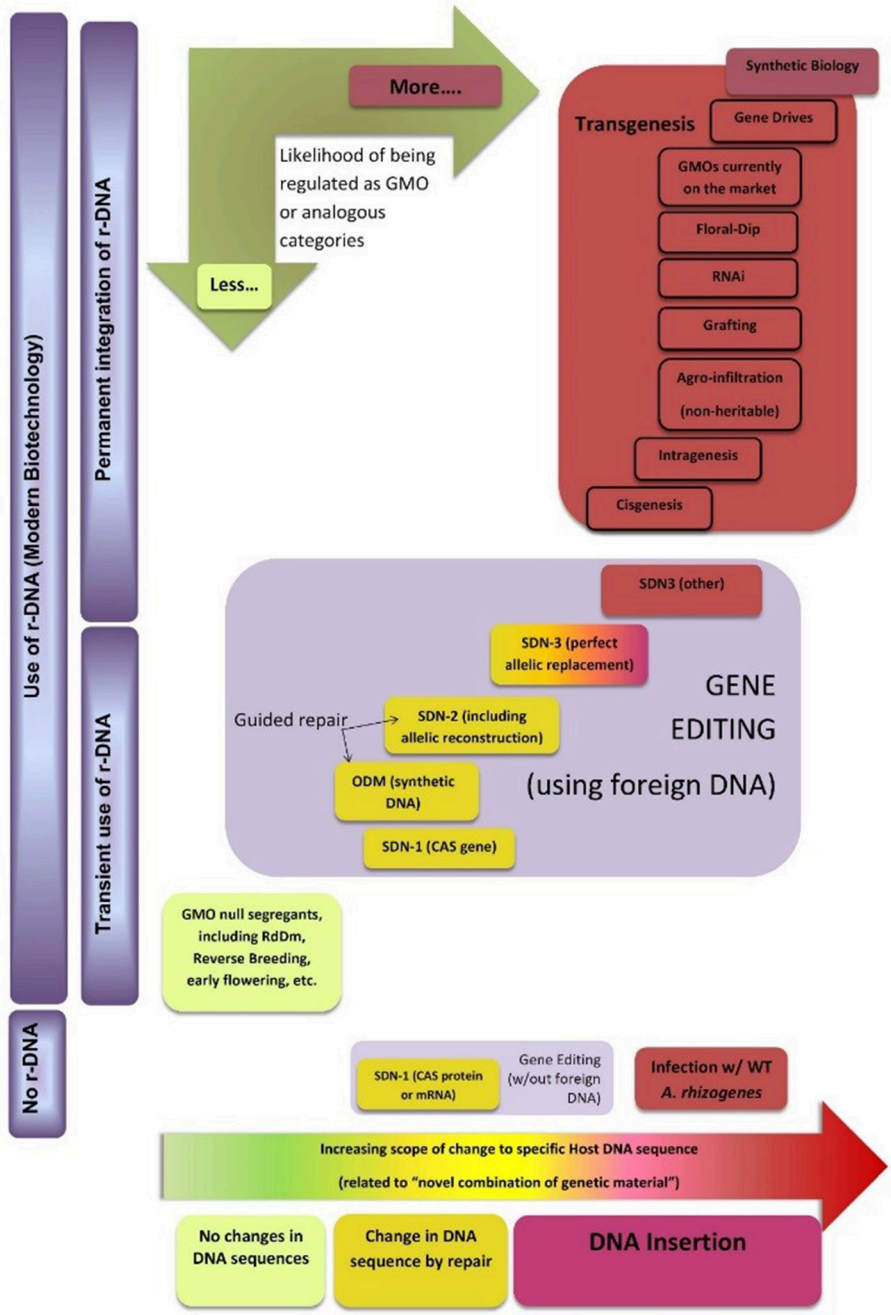

FIGURE 4 | Classification map of new breeding techniques for regulatory purposes. The authors would like to thank Dr. Huw Dylan Jones from Aberystwyth University for fruitful discussion during the elaboration of this diagram. See text for details.

European countries, as well as the preliminary policymaking discussions in the European Union and some Asian countries. In addition to these reviews, the very latest developments for an updated account of the state of play are provided next.

Argentina has issued a working regulation that has been used effectively for the last 2 years in order to establish if specific products of genome editing are GMO or conventional crops (Whelan and Lema, 2015). Recently, this regulation was extended to genome-edited animals. Chile has issued a specific regulation in 2017 (SAG)[9], and Brazil is in the process of issuing its own (CTNBIO)[10]. The three countries adopted quite similar technical

[9]SAG 82017. http://www.sag.cl/sites/default/files/RES_1523_2001.pdf
[10]CTNBIO (2018) Resolution no.16 of January 15 2018.; Brazilian Official Gazette No.15, section 1, pages 7–8. (Published January 22 2018)

criteria, both in terms of procedure (an *ex ante* assessment if the plant line is GM or not) and classification parameters (of which is paramount the presence or absence of r-DNA constructs in the genome of the line intended to be introduced in the market).

Israel also has recently issued a regulation, whose technical criteria resemble the ones applied in the Latin American countries mentioned before (Israel)[11]. Australia has launched a public consultation on GMO regulation amendments (OGTR)[12]. As a consequence of the proposed changes, some cases of genome editing may be exempted from regulation. However, the scope of potentially exempted products in Australia would be quite narrow compared to the approaches of the other countries that have made regulatory decisions until now.

Figure 4 shows a possible classification map of new breeding techniques, including genome editing, for regulatory purposes (the horizontal dimension indicates an increasing degree of intervention in specific DNA sequences of the plant that are allowed by each technology). This does not correspond to any country in particular but tries to capture emerging similarities in how the techniques seem to be perceived in different regulatory environments. It is based on the initial decisions or *ad hoc* regulations issued by some governments, as well as advice by official scientific bodies of other governments. As most countries worldwide are members of the Cartagena Protocol or use its LMO definition as a definition for GMO, its main concepts are also incorporated into the conceptual map. For practical purposes this definition encompasses two main requirements: The first one is for the organism to have a novel combination of genetic material, which can be related to the horizontal dimension as described. The second one is the use of recombinant DNA (r-DNA) to obtain such novel combination. Therefore, the map uses the vertical dimension to separate techniques that do not use r-DNA from those that use it transiently (but where it can be removed from the final organism) and those where r-DNA is permanently incorporated into the recipient genome.

As mentioned, most countries in the world regulate "GMO." Therefore, we have used this term in a wind rose incorporated to the map to indicate the likelihood with which products derived from these technologies may fall under a special regulation. The conceptual map does not include a regulatory boundary because the issue is far from being harmonized. However, it may help experts and policymakers from different countries to identify common grounds and pinpoint where exactly their differences in criteria are located, thus supporting harmonization efforts.

Product Monitoring

It has been argued that the detectability of genome edited products is technically harder compared to GMOs, and that therefore there is no point in having them regulated. In terms of policymaking, this argument is moot. Products are regulated because sectors of society want them to be regulated. If there are technical tools to detect the product the better, but if not, regulation can also be based on a system of sworn statements, traceability, etc.

For most products of genome editing, there is a clear signature in the DNA, for instance the exact stretch of nucleotides erased. If that signature is revealed by the developer, the same PCR technology used for detecting GMOs can be applied to the detection and monitoring of genome-edited products in most cases.

Conversely, there are some concerns over the possibility of new lines or breeds for which the developer does not correctly indicate the technique by which they were obtained, since (in some cases) identical changes in the DNA sequence can be generated by either genome editing or conventional breeding. Of course, detectability in this hypothetical case is more difficult, but this is also true for a genetically modified (GM) product. However, this scenario is particularly unlikely (both for GM and genome-edited organisms) as the developer would be trapped in the prisoner's dilemma because of the possibility of being "betrayed" by information released by employees, collaborators, technical publications, etc. In summary, the detectability of genome-edited products that might reach the market is not significantly different from that of GMOs and therefore, if necessary, would be covered by the already existing international instruments and technical tools.

There is certainly potential for a rugged landscape in the regulation of products from genome editing. This landscape could lead to asymmetries in the regulatory/approval status in different countries, and contribute to the "low level presence" trade issues currently experienced with GM crops (OECD, 2013). In such scenario, detection methods and other monitoring measures currently applied to GM crops will likely play the same roles and with the same efficacy in the case of gene-edited crops. The infrastructure for such monitoring and detection is already in place in many countries.

Social Issues

From the viewpoint of sociology of science and technology, genome-edited products can be regarded to be in a state of "interpretative flexibility," leaving room for discussion on whether or not they are GMO. This also means that a list of changing actors are molding the issue with evolving alliances and changing interests, and that the matter is far from being stabilized at the conceptual level.

Clearly, finding the adequate regulatory approach not only entails subjects pertaining to safety information and legal definitions; it also interplays with socio-technical resistance, international trade and innovation in agriculture. Therefore, even when the official scientific advisory bodies may advice that at least some genome-edited products should be regarded as conventional breeds or varieties from a regulatory standpoint, the political decision makers may decide otherwise for various reasons.

A relevant example of political authorities not following official scientific advice was the moratorium for GM crops in the European Union which led to a dispute in the WTO over the validity of such moratorium as a sanitary measure (Disdier

[11] Israel, 2017. Summary of National Committee for Transgenic Plants Meeting of August 8 2016 (March 2017, not publicly available, but covered e.g., here: http://news.agropages.com/News/NewsDetail-$-$22144.htm)

[12] OGTR, 2017. http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/reviewregulations-1

and Fontagné, 2010). During the case, the European Community Authorities tried to discredit the advice provided by the European Food Safety Authority (EFSA), which asserted that products were safe. This strategy aimed at justifying the governmental moratorium in GMO approvals as a sanitary measure. Finally, the work of EFSA was proven to be based on sound science using internationally agreed standards, such as the *Codex Alimentarius* Guidelines for biotechnology products (CODEX)[13].

Political decisions might result in some genome-edited products being "over"-regulated, contrary to scientific advice. It has been warned that such decisions would hamper innovation in agriculture, with potential impacts on economy and sustainability (Jones, 2015). This warning has been raised repeatedly by representatives of the academic, seed, and breeding sectors. However, these opinions have been mostly of unsubstantiated and qualitative nature.

Accordingly, as discussed in a recent article (Whelan and Lema, 2017) decision makers may need formal and quantitative studies on potential economic impacts of handling genome-edited products under different regulatory scenarios. Such studies would allow them to weigh the impact of different regulatory/policymaking options on the economy (considering trade, agroindustrial innovation and productivity). A formal analysis of the trajectory or dynamics that the interpretative flexibility is taking may be useful to anticipate the social perception of these decisions.

Private Regulations

Interestingly, as the list of social actors increases, the interpretative flexibility extends to aspects beyond sanitary regulations. For instance in Argentina and other countries there are projects applying genome editing to sport animals, such as race dogs and polo horses. This has initiated a debate in the corresponding breeding or sport associations as to whether the use of genome editing may be anti-sportive, such as unfair play or gene doping (AACCP[14]; Oliveira et al., 2011; Reuters[15]).

In conclusion, regulators and policymakers have become familiar with the technical aspects of genome editing, and debates on their appropriate regulation have sparked worldwide. These debates have extended over several years, and a wide range of actors are already involved. Some issues included in the debates, such as "product-based" vs. "process-based" regulation or product detectability initially seemed very relevant, but are not actually contributing much to decision-making. It is important to clarify that such technical debates are useful only if they help decide how to interpret and/or modify the regulatory touchstones of each country.

To date, some nations with a significant participation in international trade have already established their criteria or are close to do so. At this stage, the true remaining challenges for establishing a sound and globally harmonized regulation are more of a social than technical nature; therefore, they include an appropriate assessment of the implications of regulatory alternatives upon issues such as social perception, international trade, local innovation, and competitiveness of agroindustrial chains.

Finally, Jeffrey Wolt provided perspectives on the National Academies of Sciences, Engineering and Medicine (US-NASEM) report, which takes a step toward preparing for future biotechnology products:

In 2015, a White House Memorandum called for modernization of the biotechnology regulatory system with a focus on updating the Coordinated Framework for Biotechnology (EOP, 2015). The intent of this action was to "*clarify the roles and responsibilities of the agencies that regulate to 'products of biotechnology';*" to formulate long-term strategy for biotechnology regulatory system to efficiently assess risks "*associated with future products of biotechnology;*" to support innovation, protect health and environment, promote public confidence in regulatory process, increase transparency and predictability, and reduce unnecessary costs and burdens. The memo additionally specified "*commissioning of an external, independent analysis of the future landscape of biotechnology products*" with a focus on potential new risks and risk assessment frameworks for biotechnology products expected to emerge in the marketplace in the next 5–10 years. This effort was initiated by the Office of Science and Technology Policy (OSTP) in July 2015 and the task was undertaken by a committee of science and policy experts, convened through the U.S. National Academies of Science, Engineering and Medicine, which produced the report *Preparing for Future Products of Biotechnology* (NASEM, 2017). The committee's deliberations reflect recognition of rapid growth in the bioeconomy and the need for the U.S. regulatory system to keep pace. Their findings align with those of the U.S. Office of Science and Technology Policy's internal analysis reflecting a modernized regulatory system that effectively anticipates and addresses emerging products of biotechnology.

This contribution gives a brief synopsis of the report and introduces the implications to the emerging use of plant genome editing for crop improvement and how this may impact the ecological risk assessment process as well as regulation of future products of biotechnology.

While the report says little specifically with regard to genome-edited crops (in the view of the committee crops derived by genome editing were an existing reality for the U.S. regulatory system so represent current rather than future biotechnology products) here, a perspective as to how risk and regulatory considerations for genome-edited crops will influence the ability for innovative new biotechnologies to enter the marketplace will be provided.

Background on the Report

The rapidly changing field of biotechnology has led to innovations that were unanticipated at the time the Coordinated Framework for Biotechnology was first developed. The scope of revision of the Coordinated Framework is to address products of biotechnology more broadly and therefore, *Preparing for Future Products of Biotechnology* (NASEM, 2017) considers for its purposes "*products developed through*

[13]CODEX, 2017. http://www.fao.org/fao-who-codexalimentarius/thematic-areas/biotechnology/en/

[14]AACCP, 2017. http://www.criapoloargentino.com.ar/?sec=7&nota=965

[15]Reuters, 2007. https://uk.reuters.com/article/science-genes-dogs-dc/gene-makes-racing-dogs-fast-study-finds-idUKN0118454720070501

genetic engineering or [genome engineering or] the targeted or in vitro manipulation of genetic information of organisms, including plants, animals, and microbes." The report's key themes recognize that: (1) The "*bioeconomy is growing rapidly and the U.S. regulatory system needs to provide a balanced approach for consideration of the many competing interests in the face of this expansion.*" (2) A "*profusion of biotechnology products [envisioned] over the next 5–10 years has the potential to overwhelm the U.S. regulatory system.*" (3) Regulators will face difficult challenges that go beyond considerations of contained industrial uses and traditional environmental release as the "*safe use of new biotechnology products [will require] rigorous, predictable, and transparent risk-analysis processes that mirror the scope, scale, complexity, and tempo of biotechnology development. (4) Agencies involved in regulation of future biotechnology products would benefit from adopting recommendations made by previous National Academies' committees.*"

The urgency for a revised Coordinated Framework to address the rapidly emerging bioeconomy is evidenced in accelerants that are hastening bioengineering innovation and product development. But future products of the bioeconomy are not envisioned to reflect new risk-assessment endpoints for ecological risk assessment (ERA) and regulatory consideration; rather these products represent differing and high complexity pathways to those endpoints. Significant increases in the rate, number, and complexity of biotechnology products, and the diversity of actors involved in the research and development process, will challenge the abilities of Federal agencies. Enabling effective regulation will require streamlined access to the regulatory system in a manner which is highly transparent to developers and the public alike.

In the view of the committee (NASEM, 2017), the current Coordinated Framework for Regulation of Biotechnology appears to have considerable flexibility to address these challenges, but jurisdictional considerations have the potential to duplicate the regulatory effort or leave gaps in regulatory oversight. The U.S. biotechnology regulatory system is complex and fragmented and can be difficult to navigate. This complexity causes uncertainty and a lack of predictability for developers of future biotechnology products, which in turn has the potential for loss of public confidence in regulation of future biotechnology products. Therefore, a more streamlined, flexible and transparent system is needed.

The report concluded that U.S. "Agencies involved in regulation of future biotechnology products should increase scientific capabilities, tools, expertise, and horizon scanning in key areas of expected growth of biotechnology, including natural, regulatory, and social sciences." Additionally, pilot projects may be useful "to advance understanding and use of risk assessments and benefit analyses for future biotechnology products that are unfamiliar and complex." And finally, "agencies that fund biotechnology research with the potential to lead to new biotechnology products should increase their investments in regulatory science and link research and education activities to regulatory-science activities" (NASEM, 2017).

Perspectives Relative to Era and Regulation of Genome Edited Crops

Plant genome editing is on the leading edge of massive innovation in the field of bioengineering, which will result in diverse product types that have not been previously considered within a formal regulatory context. Assessment strategies and regulatory approaches for genome-edited crops will establish the paradigm for innovation that follows. Using approaches established for transgenic crops may hobble the abilities of regulatory authorities with knock-on effects to innovation for the bioeconomy. Therefore, there is a need to streamline and increase collaboration amongst regulatory authorities, to triage risk assessments to focus on novel/complex products, and to adopt extra-regulatory approaches to governance where appropriate.

The increasingly novel and complex products and product uses released to consumers and the environment will be difficult to monitor and recall. For instance, many genome-edited crops will be indistinguishable from varieties developed through traditional selective plant breeding, and therefore a more focused consideration of the phenotype intended for deployment will be of greater concern that the process that has been used. Enhancing capacity and capabilities for regulatory science education of scientists and of active and engaged publics will be needed. In addition, extra-regulatory research governance mechanisms should be encouraged to identify and manage risks and uncertainties earlier in the research and development process. For instance, many public institutions in the U.S. have already instituted processes within institutional biosafety committees to ensure that appropriate stepwise assessments and confinement actions are made to limit the possibility for the initiation and deployment of unintended gene drives as a result of genome editing. These and related actions can appropriately broaden the parties responsible for biosafety to encompass researchers and public parties in addition to the regulated community and regulators.

WORLD CAFÉ—INTERACTIVE SESSION ON NOVEL FEATURES TO CONSIDER

All the topics, challenges and perspectives that have been presented in the expert contributions provided a base for an interactive session, a "World Café" focused on novel features to consider that may result from the application of genome editing in plant breeding. In this session, which was led by Nina Duensing, Thorben Sprink, and Detlef Bartsch, the participants had the opportunity and were strongly encouraged to discuss some key questions of three different aspects regarding the risk assessment, monitoring and regulation of genome-edited plants. Each discussion group of approximately 20 participants rotated through all three topics, prioritizing their own and the previous groups' arguments.

Environmental Risk Assessment—Novel Demands?

In this topic, questions regarding the Environmental Risk Assessment (ERA) of genome-edited plants were discussed: Are

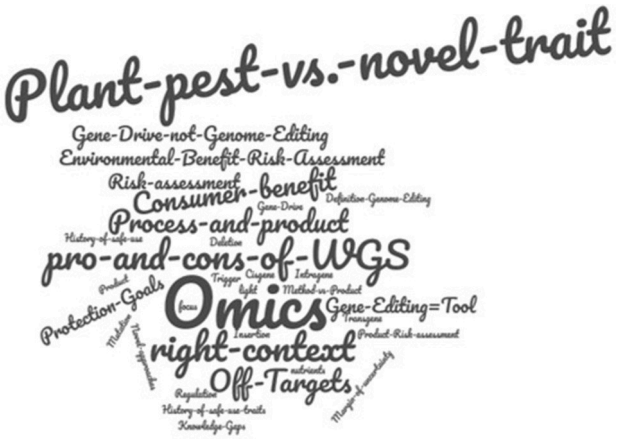

FIGURE 5 | World Café key contributions for the topic "*Environmental Risk Assessment - novel demands?"* Letter size represents the participants' ranking according importance.

FIGURE 6 | World Café key contributions for the topic "*Monitoring - detection and identification of new products after placing on the market."* Letter size represents the participants' ranking according importance.

there novel demands for the ERA of genome-edited organisms? Are there additional, novel risks to be considered, or is a simplified procedure, an "ERA light," possible? Is there a correlation of a potential risk with the modification process itself, or rather with the introduced traits? Are there potential knowledge gaps, for example the probability of additional, unintended changes ("off-target effects")? An overview of the key contributions is provided in **Figure 5**.

The most important consensus of the participants was that a combined process- and product-based approach was crucial and that all risk assessment should regard the used techniques only in the context to the modified trait. According to the majority of participants this is also true with regard to the concept of "history of safe use" which needs to be considered when performing a comparative risk assessment, as higher precision and a much lesser frequency of unintended changes are to be expected from genome editing applications. Therefore, the concept of "history of safe use" should be applied focusing on the characteristics of the resulting crop plant, not the technique used to generate them. It was reiterated that genome editing itself is only a tool; for environmental assessment, the characteristics of the final organism are decisive, not the tools that were applied to generate them.

Additionally, it became clear in the course of the discussion that the classification of genome editing applications in "site-directed nuclease" (SDN)-1, SDN-2 and SDN-3 is not generally or comprehensively defined, yet. ODM, SDN-1 and−2 are broadly seen as a targeted form of mutagenesis. Products resulting from SDN-3 are seen as GMOs, but less data may be required for their risk-assessment of cisgenic or intragenic plants than for classical transgenic plants. Furthermore, the World Café initiated a discussion on whether genome-edited plants possess specific risks for generally agreed protection goals. The general opinion was that there are no specific threats initiated by genome editing techniques. Here, again, it is crucial to restate that genome editing techniques are tools and, again, a potential risk of a plant is defined by its traits, not by the technique used in the breeding process.

Whole genome sequencing (WGS) and "-omics" tools have been a point of discussion but were generally considered of minor importance for the risk assessment. Also, gene drive applications were generally seen as less relevant for plants and for use in agricultural systems. However, noticeably, not all participants regarded gene drive systems as genome editing *per se*, and, very accurately, organisms containing engineered gene drives were generally regarded as GMOs, as these applications require the introduction of a foreign gene (i.e., Cas9). Their Regulation, risk assessment and monitoring would therefore already be coverd by GMO regulatory requirements. However, other participants mentioned that natural occurring gene drive systems, such as Medea, are already present in populations.

The World Café organizers' final conclusion of this session was that there are, in principle, no demands for a novel ERA as the existing regulatory frameworks would cover all genome-edited organisms. Instead, the improved precision plus lower probability of off-target effects as compared to conventional methods would rather simplify and focus any ERA on the introduced trait. Therefore, the adjustment of current frameworks to the increased technical precision seems appropriate.

Monitoring—Detection and Identification of New Products After Placing on the Market

In case genome-edited organisms are to be classified as GMOs and therefore be subject to GMO regulation, a monitoring system will be required for their authorization in some jurisdictions. Such a system needs specific detection and identification methods in place. Therefore, in this topics challenges and limits regarding the detection and monitoring of genome-edited organisms as well as whether or not there is a need for their detection, was discussed An overview of the key contributions to this topic is provided in **Figure 6**.

Intriguingly, there was some controversy on whether or not genome-edited organisms are, in all cases, detectable and unambiguously identifiable as such. Molecularly, small nucleotide replacements, insertions or deletions are identical, whether they occurred spontaneously, were induced by classical mutagenesis or site-specifically introduced via genome editing. Therefore, unless a foreign DNA (originating from a non-crossable, sexually incompatible organism) is inserted into a given genome, the resulting organisms will be indistinguishable from those that were developed using traditional breeding techniques. So, how shall they be monitored?

Due to the high rate of spontaneously occurring mutations and the inherent error rate of WGS applications, this tool was not considered to be an appropriate method for detection and identification of genome-edited plants. Sequence differences are expected even between close relatives or direct offspring, therefore a detected difference in any genome sequence as compared to the chosen reference genome can impossibly be attributed to a previous genome editing application. It could as well have occurred naturally or be attributed to a sequencing artifact.

It was recognized, though, that the breeders and developers themselves will have an interest for their products to be distinguishable from others. And as long as the information on the modification is provided, the modification is detectable using standard molecular biology tools, enabling an identification of the modified organism. An unknown, undisclosed modification which does not involve the incorporation of foreign sequences, however, will be hard to detect; and even if it was detected, identifying how it was introduced, i.e., by targeted mutation using genome editing tools, conventional breeding, including random mutagenesis, or naturally occurring mutations, is impossible.

While it was recognized by the participants that some form of control of genome-edited organisms seems to be desired by sectors of the public, scientifically a specific monitoring of such organisms is regarded as unnecessary, mostly because in comparison to conventional applications, a higher level of precision and safety are to be expected from genome editing. There was a high level of consensus—especially within participants from Central and South America—that the subject of detection and identification of crop plants that were produced by genome editing techniques was of minor relevance as these organisms and products thereof must not be defined as GMOs and therefore are not required to be detected or identified. The fact that it might, nonetheless, be possible that in some countries these crops might be classified as GMOs was met with incomprehension or even reluctance. This reflected the broad concurrence that the EU approach toward genome editing and other precision breeding innovations was overly restrictive and over-regulating.

The World Café organizers' final conclusion of this session was that there is no reason to establish a specific regulatory monitoring for genome-edited plants that are indistinguishable from those that were developed using traditional breeding techniques. Not only would specific monitoring requirements for basically identical varieties be scientifically unreasonable, but to require the detection and identification of such single or few nucleotide-edited organisms or products thereof would also imply an almost unsurmountable challenge to (official) analytic laboratories and enforcement institutions. In addition, the requirement of cost-effectiveness is hardly to be met even if new traceability chains would be established.

FIGURE 7 | World Café key contributions for the topic "*Harmonization of regulation*." Letter size represents the participants' ranking according importance.

Harmonization of Regulation

Free global trade requires internationally harmonized regulations. Different GMO definitions and therefore different regulation and authorization requirements would hinder international exchange—especially if the products are indistinguishable. To which extent will an international harmonization of regulation requirements be possible? Which organizations are to be responsible and able to advance and coordinate a harmonization process? Will an international consensus for regulation be possible? And if so, which consensus is favored? An overview of the key contributions is provided in **Figure 7**.

The most crucial point here was the relevance of a science-based risk and benefit analysis in order to increase public awareness and the awareness of the regulatory authorities. Various organizations' tasks and responsibilities in forwarding regulatory harmonization efforts were discussed: The integration of genome editing into the *Codex Alimentarius*[10] was considered as the most internationally useful way to provide a collection of standards for a harmonization. In contrast, the Cartagena Protocol on Biosafety to the Convention on Biological Diversity was not considered as a useful tool to advance harmonization. This sentiment may be attributed to the scope of the Cartagena Protocol which is the safe handling, transport and use of GMOs. The predominant reasoning within the vast majority of discussion participants was that organisms resulting from genome editing applications (excluding those that involve the integration of foreign DNA into the recipient's genome) are not GMOs.

Another consideration was the need to reconsider the wording of legislation documents: Instead of focusing on "risks," "safety criteria" should be the focus. The GMO definition is of central importance for an international harmonization of the regulation of genome-edited organisms. The majority of the workshop participants clearly were in favor of a process- and product-based approach, and are also in favor of an open and transparent process leading toward international coordination.

The World Café organizers' final conclusion of this session was that an international harmonization of regulation requirements is possible and urgently needed to close the risk-benefit gap between precaution and innovation potential of new genome edited organisms. It will have to be determined, which international organization can best take on this task, but failing an international harmonization will almost inevitably lead to insecurities and trade limitations. There is a need for a clear, harmonized GMO definition and for a science-based analysis not focusing merely on the potential risk but also on the benefits of the application of newly emerging biotechnology applications, including genome editing.

World Café: Summary and Conclusion

In the overwhelming majority of the 38 countries represented at the symposium, and supposedly also represented to a large extend by the workshop participants in this session (approximately 60 participants), the competent authorities pursue both process- and product-based approaches for the evaluation of genome editing and the resulting products. Given the increased efficiency and precision of these techniques, a comparably higher safety for humans, animals, and the environment is to be expected from genome-edited organisms. In many cases, a genome-edited crop variety will be indistinguishable from a variety that was developed using conventional breeding techniques. While developers might use genome editing applications to improve a plant's traits or characteristics by targeted mutagenesis, molecularly that modification in the DNA sequence will not differ from a mutation that has occurred naturally or through conventional mutagenesis. Therefore, a general classification as GMO (under the current GMO definition), including all regulatory, detection and monitoring requirements, is not desired and not seen as scientifically justified or practically enforceable.

A globally harmonized regulatory approach is considered highly important and might, in principle, enable the linkage of innovation and precaution. There was a general agreement that products resulting from genome editing will reach the market, and to date some crop plant varieties are already being commercially produced in the U.S. and a few other countries. A potentially emerging solely product-based regulation in some countries may cause trade issues, not only for genome-edited crops but also for conventional breeding products.

There was a debate of whether a new regulatory framework is needed for products resulting from genome editing or if the already existing frameworks are adequate. If a novel framework was needed, could there be a science-based risk assessment for genome-edited products? How could such a risk assessment look like? What should be included? These questions were intensely discussed and further points of contention which will have to be addressed in the future were identified: To date, a broadly accepted definition of what is considered "natural" in a regulatory context is missing or inconsistent, and a definition of "recombinant nucleic acid" is lacking, leaving spaces for interpretation. There is also a dissent in the possible regulation of products resulting from SDN-3 approaches using self-cloning and whether or not it is possible to detect and identify the products of genome editing solely by the product itself.

The World Café organizers' final conclusions were that no new regulatory frameworks for genome-edited plants are considered necessary. Existing frameworks are still adequate but may need adjustments, for example concerning a decrease in data requirements due to the increase in precision, and if comparability with already existing—safely used—non-GM and GM organisms allows this. Also, if a genome-edited plant is indistinguishable from a variety that was developed using traditional breeding techniques there is no scientific reason to call for a specific regulatory monitoring of this plant. Finally, international regulation should allow for a flexible handling of constantly emerging scientific progress. This requires an internationally harmonized GMO definition and a flexible adaptation of regulation to technological progress, taking into account appropriate scientific supervision.

CONCLUSION

Increasing technical efficacy and decreasing costs revolutionize the tools that science-driven economies can apply to increase a crop's genetic variability, a major resource for plant breeding. This high efficacy and low cost, however, could be rendered useless if appropriate regulation is not established to provide a framework that enables the use of these new tools. It is time and opportunity to find the right balance between precaution and innovation for the benefit of plant breeding. Risk assessment and regulation need to balance the public's need for food, feed, and environmental safety with the costs for developers, growers, shippers and processers without wasting resources and in a proportionate, science-based way. This requires an international harmonization of regulatory frameworks, and while there is currently no demand for a novel ERA for genome-edited organisms, adapting the existing frameworks to the increased technical precision as compared to conventional methods seems appropriate.

DISCLAIMER

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 they work for.

AUTHOR CONTRIBUTIONS

ND, TS, and DB wrote, checked and edited the manuscript. WP, TS, MF, ML, and JW contributed the sections on their respective talks. ND, TS, and DB contributed the sections on the interactive session. All authors have read and approved the manuscript for publication.

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Evaluation of the Impact of Genetically Modified Cotton After 20 Years of Cultivation in Mexico

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For more than 20 years cotton has been the most widely sown genetically modified (GM) crop in Mexico. Its cultivation has fulfilled all requirements and has gone through the different regulatory stages. During the last 20 years, both research-institutions and biotech-companies have generated scientific and technical information regarding GM cotton cultivation in Mexico. In this work, we collected data in order to analyze the environmental and agronomic effects of the use of GM cotton in Mexico. In 1996, the introduction of Bt cotton made it possible to reactivate this crop, which in previous years was greatly reduced due to pest problems, production costs and environmental concerns. Bt cotton is a widely accepted tool for cotton producers and has proven to be efficient for the control of lepidopteran pests. The economic benefits of its use are variable, and depend on factors such as the international cotton-prices and other costs associated with its inputs. So far, the management strategies used to prevent development of insect resistance to GM cotton has been successful, and there are no reports of insect resistance development to Bt cotton in Mexico. In addition, no effects have been observed on non-target organisms. For herbicide tolerant cotton, the prevention of herbicide resistance has also been successful since unlike other countries, the onset of resistance weeds is still slow, apparently due to cultural practices and rotation of different herbicides. Environmental benefits have been achieved with a reduction in chemical insecticide applications and the subsequent decrease in primary pest populations, so that the inclusion of other technologies—e.g., use of non-Bt cotton- can be explored. Nevertheless, control measures need to be implemented during transport of the bolls and fiber to prevent dispersal of volunteer plants and subsequent gene flow to wild relatives distributed outside the GM cotton growing areas. It is still necessary to implement national research programs, so that biotechnology and plant breeding advances can be used in the development of cotton varieties adapted to the Mexican particular environmental conditions and to control insect pests of regional importance.

Keywords: Bt cotton, center of origin, environmental impact, GMO, herbicide, Mexico

INTRODUCTION

Cotton is one of the most important natural sources for fiber, oil, and seeds for livestock feeding. All the cotton produced in the world is obtained from four domesticated species of the *Gossypium* genus of the Malvaceae family. With 18 species, Central, and South America are the richest regions in *Gossypium* species globally, being Mexico one of the most diverse countries with 14 different species. The northeast of Africa and the southwest of Arabia also have 14 different species and Australia has 17 species (Cronquist, 1981; Fryxell, 1992; Percival et al., 1999).

An outstanding feature of cotton domestication is that it occurred simultaneously in different continents from local cotton wild ancestors. This process of parallel and convergent domestication occurred for the species *Gossypium hirsutum* in Mexico, *G. barbadense* in Peru, *G. arboreum* in Sudan and *G. herbaceum* in Pakistan. In each of these four cases, the unique properties of cotton fiber useful to make ropes and textiles were noticed thousands of years ago. From these four species, *G. hirsutum*, commonly referred to as Mexican cotton or highland cotton, is the most widely planted, accounting for 90% of world production. This is relevant, since Mexico is an important center of origin and domestication of many other cultivated crops, such as corn, squash, pumpkin, bean, and chilies. Currently in Mexico several native cotton species are present, including the wild relatives of *G. hirsutum*. The highest concentrations of wild cotton relatives are located in the southeast region of the country, the only place where *G. hirsutum* is found as a common species in the native flora (Coppens d'Eeckenbrugge and Lacape, 2014).

Before the deployment of GM technology, cotton production was associated to high environmental, economic, and sanitary costs due to the necessity of large amounts of pesticide applications. A different strategy was necessary to improve yields, thus technology involving GM cotton cultivars with inserted genes that confers resistance to lepidopteran pests and to herbicides was adopted by the growers (Deguine et al., 2008; Benbrook, 2012).

In Mexico as in other parts of the world, the cultivation of cotton was characterized by the application of large quantities of chemical insecticides. For example, in the 1970s decade, cotton cultivation required almost 20 applications of chemical insecticides from the plant emergence to harvest, since cotton plants must be protected from insect attack when the plant emerges, until the profitable bolls open (a period that lasts ∼20 weeks). In the middle of the Twentieth century, at the peak of cotton production in Mexico, the cotton area that was planted reached 900,000 hectares with 2 million bales produced per year (the term "white gold" was used at that time to describe cotton). Years later, the increasing pest pressure and high doses of pesticides resulted in the evolution of insect resistance to chemical insecticides. In addition, reductions in international prices of the fiber resulted in a production decline due to unsustainable operating costs (Martínez-Carrillo and Díaz-López, 2005; Martínez-Carrillo, 2015).

In 1996, GM cotton was for the first time commercially planted in Mexico as well as in five other countries (James, 2016), due to the impossibility of cultivating conventional cotton in areas of severe pest pressure (Terán-Vargas et al., 2005). Since then, a total of 15 countries have commercialized GM cotton (Argentina, Australia, Burma, Brazil, Burkina Faso, China, Colombia, Costa Rica, United States, India, Mexico, Paraguay, Pakistan, South Africa, and Sudan). In Mexico, the increase in GM cotton adoption was gradual (Martínez-Carrillo, 2005), and since 2008 the 96% of the area cultivated with cotton was GM cotton (Purcell et al., 2008).

Nevertheless, the area planted with GM cotton in Mexico has fluctuated, depending on international fiber prices, input costs and the prevalence pests, weeds, and diseases. The main cotton production areas of Mexico are located in the northern region of the country. This region has an arid climate and growers used irrigation systems. These areas of cotton production are not in close proximity to areas containing wild relatives of cotton, as stated in the Mexican law (CIBIOGEM, 2018).

The transformation events or transgenes that have been authorized in Mexico since 1996 confer two main traits, one is the tolerance to herbicides and the other is the resistance to lepidopteran pests. In the first case, plants are tolerant to herbicides such as glyphosate (Nida et al., 1996), ammonium glufosinate (Blair-Kerth et al., 2001) and dicamba (Cahoon et al., 2015) that are used to combat weeds. In the second, resistance to lepidopteran pests is due to the insertion of *cry* genes from the bacterium *Bacillus thuringiensis* (Bt) that confers resistance to larval stages of different lepidopteran pest such as *Pectinophora gossypiella*, *Helicoverpa zea*, *Heliothis virescens* (Benedict et al., 1993), and *Spodoptera exigua* (Wilson et al., 1992; James, 2016).

In Mexico, the "Biosafety Law of Genetically Modified Organisms" regulates the cultivation of GM cotton and other biotech crops in a step-by-step and case-by-case basis. The different steps refer to the different stages of release: experimental, pilot and commercial plantings. Prior to the commercial release, the authorities evaluate the results of the experimental and pilot (semi-commercial) scale releases, carrying out risk assessment studies and examining the experimental results, as well as the compliance and effectiveness of the biosafety measures (DOF, 2005). Academic institutions must endorse the research carried out in Mexico. A total of 15 GM cotton release events were requested from 2005 to 2015, in 342 dossiers [**Figure 1**; (CIBIOGEM, 2018)].

The environmental risk assessment studies aim to identify potential damage to the environment where the level of risk is estimated, the potential negative effects are identified, and actions needed to reduce environmental risks are determined (EPA, 1998). In the case of the environmental risks associated with the release of agricultural GMOs, it is important to compare them with the risks associated to the agricultural practices used on conventional crops. This is why a "case by case" analysis should be performed, that is, to consider the modified organism, the intended use, and the likely environment and environmental conditions in which it will be grown. The risk assessment studies for the release of GM cotton in the case of Mexico included an evaluation of the risks of gene flow to wild relatives, the

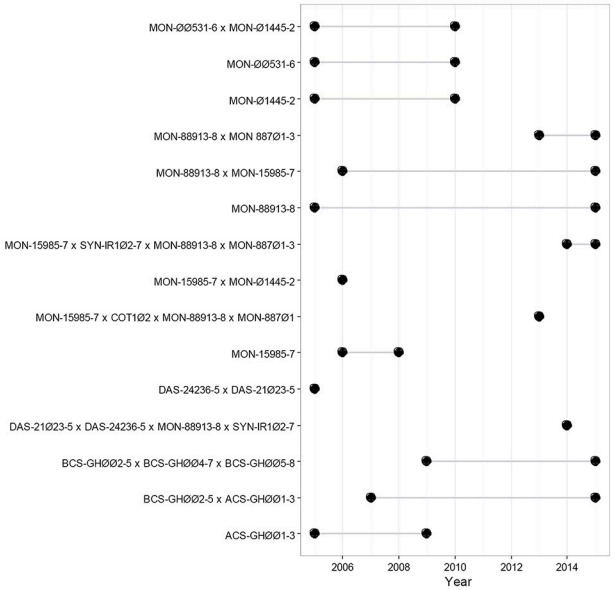

FIGURE 1 | Timeline for GM cotton transformation events released in Mexico. Black dots indicate the first and last year that each release was requested. The figure shows the different GM cotton released applications that have received a permit in Mexico, from 2005 until 2015 (CIBIOGEM, 2018).

possible effect on non-target organisms, the risks of selection of resistant weeds to herbicides and the evolution of resistance to Cry proteins by the insect pests (SEMARNAT, 2018).

In this work, we present an updated analysis of the data available since the release of GM cotton in 1996. Two main hypothesis were questioned: the first hypothesis is if there is potential risk in gene flow to native species, while the second is if the use of GM-cotton in Mexico would result in a reduction of pesticides use and in higher yields.

METHODS

Analysis of Wild Cotton Species Distribution

For the analysis of the wild cotton species distribution, we used the CONABIO database where 2,238 records were cured and verified (including 16 cotton species: *G. thurberi, G. armourianum, G. harknessii, G. davidsonii, G. aridum, G. raimondii, G. gossypioides, G. lobatum, G. laxum, G. trilobum, G. turneri, G. schwendimanii, G. lanceolatum, G. hirsutum,* and *G. barbadense*; CONABIO, 2018).

In order to assess the likelihood of gene flow, the cotton growing regions were characterized and a distribution model of wild *G. hirsutum* was constructed. The environmental characteristics of these cotton growing regions were identified by a classification tree, using as covariates of 19 current bioclimatic layers (Hijmans et al., 2005), 12 solar radiation layers (WorldClim), terrain slopes and ruggedness index.

Development of an Ecological Niche Models

To elaborate ecological niche models (ENM) of two different scenarios of cultivated cotton (without volunteers and with volunteers), we used a database constructed with 259 unique presence records of GM cotton plots and 17 records of cotton volunteers reported by several volunteer monitoring campaigns carried out in the cotton growing regions. Records from plots in the Northeast region (Tamaulipas) were not available and were not included in the analysis.

Nineteen current bioclimatic layers were downloaded from the WorldClim 1.4 data set (Hijmans et al., 2005) and six topographical layers from the HYDRO1k Elevation Derivative Database (available at: http://lta.cr.usgs.gov/HYDRO1K), using a resolution of 30 arc-s (ca. 1 km).

Maxent 3.3.3e (Phillips et al., 2006) runs were performed, one for each scenario. Each run included 30 replicates using the logistic model, and 20% random test by bootstrap. All the distribution models were evaluated using AUC scores (0.98 with and without volunteers). The models were transformed into binomial data, with a total presence value as the cut-off for each scenario (0.01 without volunteers and 0.15 with volunteers).

Surveys of Cotton Farmers

In Mexico, cotton farming is commonly managed by the owner of the land or the farmer that uses it, and a "technical advisor," that is a professional pest control crop advisor.

In order to determine the perception of the Mexican farmers on the impacts of planting of GM cotton, a survey was designed and applied to 167 farmers in 20 municipalities of the main cotton-producing states. The objectives of the survey were to identify factors associated with the use of GM cotton in Mexico, to know the willingness of farmers to use this biotechnology and the perception of benefits or problems that they have observed, to identify changes in yields, production costs, control of pests, handling, and use of pesticides from the transition from conventional to GM cotton and to evaluate the indirect effects of the use of this technology on the environment and in human health. The survey was designed according to the methodology of agricultural surveys with multiple sampling frames and the sample design for the study of rural organizations in Mexico (Kish, 1990; González-Villalobos and Wallace, 1998). The margin of error of this survey was ±7.46% with a total estimated population of 5,000 cotton farmers and a confidence level of 95% (Survey System, 2018).

Surveys of Technical Advisors

A survey was applied to 165 technical advisors specialized in cotton management. This survey was based on Shaw et al. (2009), to assess the impact of GM-crops with tolerance to glyphosate. Questions related to the pest management were also added.

The technician advisors' sample size was: Mexicali ($n = 46$); Chihuahua ($n = 39$); and La Laguna ($n = 80$) (**Figure 2**). The margin of error of this survey was ± 7.5% and a confidence level of 95% (Survey System, 2018).

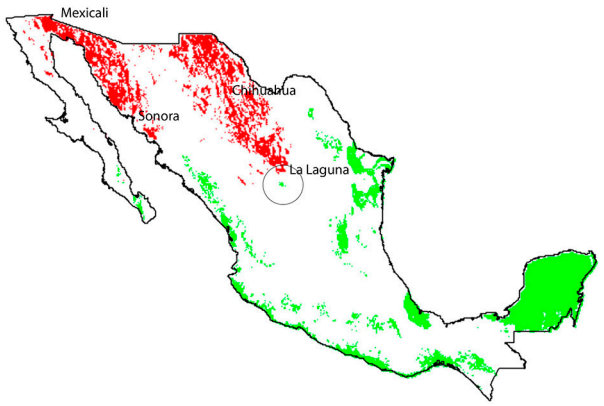

FIGURE 2 | Climatic suitability model for geographical space projection regions for wild cotton *G. hirsutum* (green dots) and cultivated GM cotton regions (red dots). Regions do not overlap, but show proximity in the area known as "La Laguna" (black circle).

RESULTS AND DISCUSSION

Exploring the First Hypothesis: Gene Flow From Cultivated Cotton (Conventional or Transgenic) to Wild Relatives

Since Mexico is a center of the origin and diversification of *G. hirsutum*, one of the main environmental concerns for the release of GM cotton was the possibility of transgene flow to native cotton populations (Ellstrand, 2002, 2012; Ellstrand et al., 2013).

In Mexico there is a continuum of *G. hirsutum* cotton varieties that range from wild, feral and locally domesticated to improved varieties, therefore the potential for gene flow among them exists if they coexist in the same area. To assess such risk, it is necessary to know the geographic distribution patterns of the different varieties, and also the dispersal mechanisms of the species. The geographical distribution of wild populations and cultivated cotton was taken into account in the risk assessment evaluation and the geographical separation constitutes one of the conditions in México for the release of GM cotton into the environment and before sowing field visits were done to identify the possible presence of wild cotton relatives (BCH, 2018; SAGARPA, 2018).

The geographical overlap between native species distribution and the region in which GM cotton is currently planted is minimal, according to the records of the "National Commission for Knowledge and Use of Biodiversity" (CONABIO, 2018). The delimited GM cotton growing regions correspond to semi-arid regions (**Figure 2**, red dots) that do not geographically overlap with the area of climatic suitable zones of wild *G. hirsutum*. However, they are close to the La Laguna region (**Figure 2**). Few GM cotton regions were not included in the analysis either due to security issues or restrictions in technical support (i.e., North of Tamaulipas, Valleys of Yaqui, and Mayo, and Planicie Huasteca), all of them coincided with the climatic suitability zones of *G. hirsutum*. Nevertheless, according to the National Statistics (INEGI, 2012), Tamaulipas is the state with less cotton production in the country and the Yaqui valley as well as the Planicie Huasteca are not even in the statistics of cotton production.

For gene flow through pollen to occur, it is not only required that the plants coexist in the same area and that they are compatible, but also that the pollen containing transgenes is dispersed via pollinators. In the case of cotton, the rate of cross-pollination (the probability that a plant is pollinated with pollen from other plant) is 10% or less, since 90% of the plants resulted from self-pollination (Meredith and Bridge, 1973; Llewellyn and Fitt, 1996; Sen et al., 2004; Van Deynze et al., 2005; Zhang et al., 2005). It was also reported that, in cases where cross-pollination by bees occurs, it significantly decreases with the distance between plants. High cross-pollination probability occurs only when plants are located in close proximity (Umbeck et al., 1991; Yan et al., 2015). Moreover, the cross-pollination rate depends, to a large extent, on the climatic and ecological condition that determine, for example, the patterns of activity and abundance of insect species carrying out pollination and pollen flow (Llewellyn et al., 2007).

However, in our study we observed that the most imminent risk of gene flow is not by pollen, but by seeds spilled during transportation. Cotton-seeds can be efficiently dispersed by either wind or water. During several field visits to the cotton productions areas, it was observed that there is a very strict control and biosafety measures during the movement of the GM cotton-seeds from the seed-companies to the fields. The GM seeds arrive in closed packages and closed vehicles. However, after the harvest, such controls relaxed, and the seeds are transported to the gins in open vehicles that spill seeds in the roads. Volunteer plants can grow from spilled seeds and have been observed in the edge of roads. Sanity authorities and seed companies are in charge of removing the volunteer plants, but unnoticeable escapes are always possible.

From the two scenarios of cultivated cotton (without volunteers and with volunteers), we further elaborate an ENM as described in Methods. **Figure 3** shows the Principal Component Analysis (PCA) of environmental conditions of the analyzed cotton records (wild, GM, and volunteer). It can be seen that the conditions in which GM cotton is planted (blue dots) are very restrictive and conditions are clearly differentiable from the rest of the cotton species (wild in black, gray, and colors). However, the presence of GM volunteers (red dots) in environments other than GM growing regions demonstrates the environmental plasticity of GM cotton, and broadens the environmental component of the GM cotton niche toward the environmental space occupied by wild species. In **Figure 4** we show the potential distribution of GM and wild cotton. According to the models describing the two possible scenarios (without and with volunteers), this figure shows that the presence of volunteers significantly expands the niche of GM cotton in its geographic component (**Figure 4**).

It is important to mention that Wegier et al. (2011) reported the existence of gene flow at long distances between cultivated and wild populations of *G. hirsutum*, by the identification of recombinant proteins in wild populations of cotton. These authors proposed that the gene flow may be possible through the

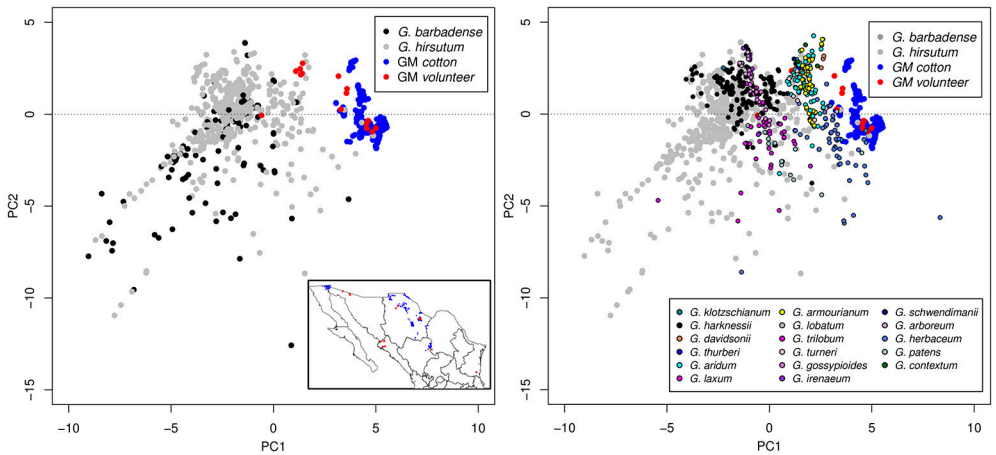

FIGURE 3 | Principal Component Analysis (PCA) of environmental conditions of the analyzed cotton records (wild, GM, and volunteer). Conditions in which GM cotton is planted (blue dots) are very restrictive and differentiable from the rest of the cotton species (wild in black, gray, and colors). GM volunteers are represented with red dots.

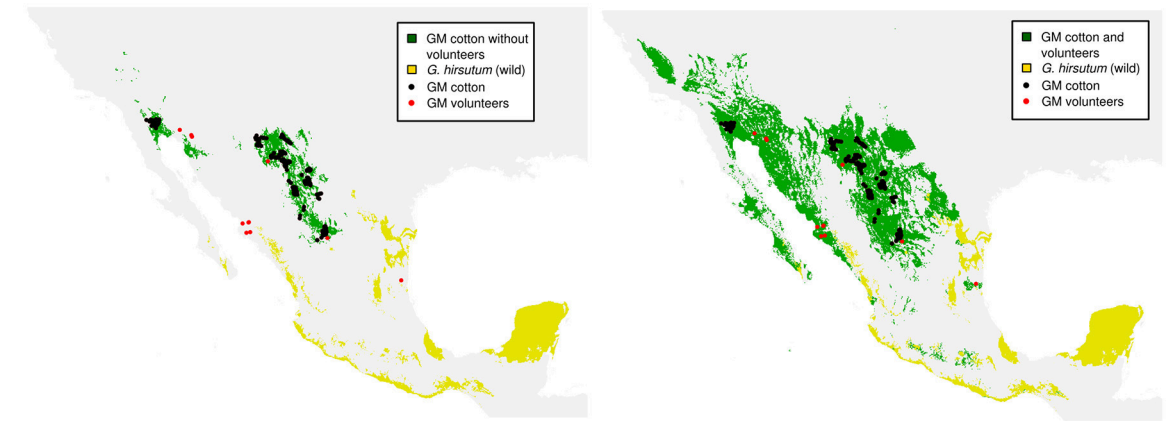

FIGURE 4 | GM cotton distribution models adding the presence of volunteers. In green: potential distribution of GM cotton. In yellow: potential distribution of wild cotton. Black dots indicate the records of GM cotton plots while red dots indicate the records of volunteer plants used for the elaboration of the models.

dispersion of seeds (Wegier et al., 2011). Hence, it is necessary to follow up the monitoring of hybrid populations and implement sensitive methods such as RT-PCR and digital-PCR to evaluate in detail the changes in transgene frequencies in these populations (Holst-Jensen, 2009; Fraiture et al., 2015; Randhawa et al., 2016).

What Do People That Work With the GM Cotton in Mexico Think

Surveys of Cotton Farmers

Overall, farmers pointed out that the use of GM cotton resulted in better pest control and easier pest management. Also, higher yields of GM cotton were generally mentioned. The reasons for stopping the planting of non-Bt conventional seed include difficulty for controlling pests and high costs of insecticides. According to the opinion of the farmers, GM cotton showed higher yields and required less use of insecticides and crop management. Nevertheless, according to farmers' opinions GM cotton-seeds are expensive and the use of herbicides is higher. In addition, farmers agreed that the highest yields of GM cotton are due to better seed quality and favorable weather conditions.

Cotton is planted in the arid areas of northern Mexico, where adverse weather conditions are prevalent, including the lack of water, extreme temperatures, drought, and frost. Inputs such as special planting equipment, irrigation, and fertilizers result in high production costs. In addition, an increase in seed prices, machinery, and fuels in recent years exacerbated the production costs.

The high operation costs as well as fluctuations in international fiber prices, led to a large fluctuation in the total cotton area planted. For instance, in 2016 the total cotton area in Mexico was reduced to 104,000 ha, due to the decrease in international prices and the increase in input costs. However, the cotton area was doubled to 210,000 ha in 2017 due to an increase in international fiber prices. The decrease in grain prices could

be another important factor that favors cotton growing for some farmers.

Despite the cost of production, 80% of the farmers are highly satisfied with the use of the GM varieties, since the lepidopteran pests are controlled and excellent weed control is obtained. The remaining 11% of farmers are moderately satisfied, and 9% are not satisfied. Ten percent of the farmers considered that GM cotton is not profitable.

Interestingly, 40% of the farmers would be willing to plant conventional seeds if available in Mexico (conventional seeds are not produced now in Mexico), because it is assumed by these farmers that those seeds would cost less. Furthermore, due to current pest populations observed for the past few years, they considered that current pests are not necessarily controlled by GM varieties.

From the point of view of the effects on human health, farmers have a positive perception about the adoption of GM cotton. They believe that the intoxication cases due to chemical pesticide exposure have been reduced with the adoption of GM cotton. They reported less intoxication cases due to a lower use of chemical insecticides (Nava-Camberos et al., unpublished results).

Surveys of the Technical Advisors

In order to analyze changes in pest and weed management after the adoption of GM cotton a survey was applied to 165 technical advisors specialized in cotton management.

With respect to the management of weeds and herbicides, the responses of the technicians indicated that glyphosate is practically applied to the entire cotton growing area in Mexico at least once during the production cycle. The main weed species associated with cotton are field bindweed *Convolvulus arvensis L.*, annual morning glories *Ipomoea hederacea* Jacq. and *Ipomoea purpurea* (L.) Roth, palmer amaranth *Amaranthus palmeri S.* Wats, johnsongrass *Sorghum halepense* (L.) Pers. and various annual grasses, mainly barnyardgrass *Echinochloa colona* (L.) Link.

According to these surveys, weed management in cotton in Mexico generally consists of the application of glyphosate that is complemented by deep tillage for soil preparation and in-row cultivation in more than 90% of the cotton area. The application of other herbicides in addition to glyphosate in pre-planting and pre-emergence is done in about 21% of the area where trifluralin represents the most used herbicide in these early applications.

Technicians indicated that problems associated with weed management were reduced in Mexicali and La Laguna, but they were increased in the state of Chihuahua, where the control of weeds with glyphosate was qualified as low. Sixty two percent of the technicians indicated that they have observed changes in the response of weeds to glyphosate. This response of the weeds implies the need of a dose increase of herbicides in order to have an effective control in the most difficult weeds. Nevertheless, 85% of the technicians are currently carrying out management practices to prevent the selection of glyphosate-resistant weeds, focusing mainly to in-row cultivation, hand weeding and crop rotation.

Before the use of Bt cotton, the Lepidoptera complex (*P. gossypiella*, *H. zea*, *H. virescens,* and *S. exigua*) comprised the majority (ca. 60%) of the total reported pests; followed by sucking insects (whitefly, *Chlorochroa ligata* and *Lygus*; ca. 20%). The reported insects list is presented in **Table 1**, where it is observed this drastic drop in lepidopteran counts, while other insects such as aphids, mites, weevils, thrips, and whiteflies increased in counts by the technicians. The technicians consider that the pressure of the Lepidoptera complex was very high before the use of GM-cotton and now it has effectively been reduced.

After 20 years of using Bt-cotton, the interviewed technical advisors have observed drastic changes in the composition of insect pest species. Currently, the most important are *Anthonomus grandis*, *C. ligata*, *Bemisia tabaci*, several species of sucking insect pests, and thrips. The Lepidoptera complex represented only up to 5% of the reported pests (mentioned by 0, 0, 0, and 5% of the technical advisors in Mexicali, Sonora, La Laguna, and Chihuahua, respectively) while the sucking insect pests comprised around 73% (60, 60, 80, and 95% of the survey in Sonora, La Laguna, Chihuahua, and Mexicali, respectively). Due to environmental differences in the cotton growing regions of Mexico, it is difficult to rank the overall importance of pests. For example, whiteflies are of primary importance in Mexicali, Sonora, and La Laguna, but in Chihuahua, they are considered a secondary pest. Conchuela (*C. ligata*) is still considered the primary pest in La Laguna and Chihuahua, but it is not a concern in Mexicali and Sonora. *A. grandis* once a menacing pest throughout Mexico, currently is only important in La Laguna and Sonora, but in Mexicali and Chihuahua this pest is eradicated. This eradication is due to the joint *A. grandis* eradication Mexico-USA program. After using Bt-cotton, *P. gossypiella*, *H. virescens*, and *Bucculatrix thurberiella* now have very low population levels in the different cotton regions. *H. zea* and *S. exigua* are currently considered pests of secondary importance in all cotton areas (**Table 1**).

Regarding the number of total insecticide applications, the technicians reported a significant decrease due to the use of GM cotton. Due to the effectiveness of Bt-cotton, and its high rate of adoption in most of the growing areas, in Chihuahua and La Laguna the synthetic insecticides sprays have been reduced to 3.5 and 5.0 applications, respectively, from the previous ∼12 applications used in a crop season. Nevertheless, in other regions such as Mexicali and Sonora that showed high pressure of pests that are not targeted by Bt-cotton (whiteflies, *Lygus* bugs, and boll weevils) the insecticide sprays are still high.

Exploring the Second the Hypothesis: Effects and Impacts of GM Cotton Cultivation in Mexico

Different lines of evidence indicated that the use of GM cotton has contributed to reducing the number of insecticide applications necessary to achieve adequate control of lepidopteran pests in the cotton regions of Mexico. Cotton is one of the crops in which the greatest amount of pesticides is applied in the world, so the alternative of using Bt-cotton represents an advantage from the environmental point of view (Abedullah et al., 2015). It is known that the use of pesticides can have negative impacts on the quality of water and soil, human health, aquatic species, and beneficial insects and other

TABLE 1 | Insects reported by technicians as important pests in all regions.

Insects reported by technicians	Before	After	Effect
DECREASE IN COUNTS			
Cotton bollworm (*Helicoverpa zea*)	116	6	−110
Pink bollworm (*Pectinophora gossypiella*)	103	6	−97
Beet armyworm (*Spodoptera exigua*)	17	7	−10
Cotton leaf perforator (*Bucculatrix thurberiella*)	6	0	−6
Tobacco budworm (*Heliothis virescens*)	3	0	−3
Fall armyworm (*Spodoptera frugiperda*)	2	1	−1
Cabbage looper (*Trichoplusia ni*)	2	1	−1
Cotton fleahopper (*Pseudatomoscelis seriatus*)	1	0	−1
INCREASE IN COUNTS			
Yellow sugarcane aphid (*Melanaphis sacchari*)	0	1	1
Stink bug (*Nezara viridula*)	1	2	1
Red spider mite (*Tetranychus* sp.)	0	3	3
Cotton aphid (*Aphis gossypii*)	3	13	10
Boll weevil (*Anthonomus grandis*)	46	57	11
Conchuela bug (*Chlorochroa ligata*)	23	47	24
Other Hemipterous plant bugs	23	50	27
Thrips (*Frankliniella occidentalis* y *Thrips tabaci*)	4	33	29
Lygus bug (*Lygus lineolaris*)	5	38	33
Whitefly (*Bemisia tabaci*)	21	71	50

Numbers indicated the number of times that a technician mentioned the name of the insect as important before the deployment of Bt cotton and after it. The effect was measured by the subtraction of both values. Negative values indicate a decrease in the number of times reported and positive values indicate an increase in the times reported.

organisms (Boatman et al., 2004; Arias-Estevez et al., 2008; Athukorala et al., 2012).

According to most farmers, GM cotton in Mexico, despite its costs, is still economically profitable and is one of the main income sources in the municipalities where it is planted. In those places, GM cotton seems to ensure production, and prevent losses by lepidopteran insect pests, while reducing costs and labor activities as well as the use of vehicles to spray pesticides (Skevas et al., 2013). The impact on crop yield has also been significant since in Chihuahua, La Laguna and Mexicali the yield increments are 1.8, 2.4, and 3.7 bales per ha, respectively, which is equivalent to increases of $ 8,700, $11,500, and $17,700 Mexican pesos per ha.

It is difficult to illustrate the agronomic advances that the cotton industry has experienced in recent decades without also involving factors such as the improvement of seeds, the better use of water and fertilizers. Great effects are the result of better training of the agricultural technicians and government campaigns for crop health. Pest eradication is an additional benefit of this technology. For example, since 2007 it has not been necessary to apply insecticides against *P. gossypiella* in Chihuahua. It is calculated that the *P. gossypiella*-eradication program resulted in 1.7 million less liters of chemicals saving of more than 207 million Mexican pesos for cotton producers (CESAVECH, 2015).

Few studies have analyzed the effect on human health and the environment of GM cotton. Adoption of Bt-cotton reduced acute pesticide poisoning in farmers in China and India (Hossain et al., 2004; Kouser and Qaim, 2011). The compounds present in the pesticides used in conventional crops tend to accumulate in human tissues and are very dangerous for workers if the appropriate safety equipment is not used.

As mentioned before, different data and our surveys indicate that the intensity with which pesticides were used before GM cotton was very high. The intense use of broad-spectrum insecticides in conventional cotton was highly toxic, since those compounds affect many kinds of animals, including humans, and usually have high permanence in the field, affecting food chains of predators, parasitoids, and pollinator insects.

Ecological and Evolutionary Aspects of GM Cotton

Effect of Bt-cotton on Non-target Insects

Annual crops such as cotton require a field season comprised of 6–7 months and involve the intensive management of both weeds and insect pests. The Cry toxins produced by Bt that are expressed in different cotton events (Bt cotton) are specific to insects of the order Lepidoptera. These toxins are active against common cotton pests such as *P. gossypiella*, *H. zea*, *H. virescens,* and *S. exigua*. Thus, the control of other pests of different insect orders that attack cotton such as the coleopteran *A. grandis*, or the hemipteran *B. tabaci* or other insect pests still require applications of synthetic insecticides.

It is important to note that formulated insecticides based on Bt are used in integrated pest management (IPM) and organic agriculture because of their high specificity. Bt is also integrated into pest management, due to its biodegradable nature and ability to control specific pests, lacking impact on non-target organisms such as bees, parasitoid wasps, earthworms, beneficial true bugs, or predatory beetles, which do not possess an active target site (or receptor) where the Bt protein can interact (Pardo-López et al., 2013). The results of numerous studies with Bt toxins show that when non-target organisms are exposed to Bt toxins in similar amounts, or higher than those produced by the Bt-crops, they are not affected (Zwahlen et al., 2003; Ferry et al., 2005; Lu et al., 2010; Schuler et al., 2013). Among the most detailed studies are those in which a pest (e.g., an aphid, mite or worm) is fed on Bt-cotton and is consequently consumed or parasitized by a predator/parasitoid without any effect on the non-target insect (Zwahlen et al., 2003; Ferry et al., 2005; Lu et al., 2010; Schuler et al., 2013).

Due to the high effectiveness of Bt cotton against the most important lepidopteran pests, the damage induced by these Lepidoptera complex in Bt cotton is substantially smaller, or non-existent, when compared with the damage that they produced on conventional cotton if they were not controlled by chemical insecticides. However, the reduction of lepidopteran pests in Bt cotton may result in an increase of other cotton pests that are not controlled by Bt cotton. This phenomenon has been observed worldwide (Wang et al., 2006, 2009; Zhao et al., 2011) suggesting that secondary pests can occupy the resources previously used by lepidopteran insects. However, it was also reported that the lower use of chemical insecticides promotes the increase of natural enemies than can decrease populations of other non-target pests (Tian et al., 2015).

This increase of secondary pests apparently has been erroneously interpreted as an undesired effect of Bt cotton (Wang et al., 2008; Li et al., 2011; Zhao et al., 2011). Nevertheless, farmers generally control outbreaks of secondary pests with broad-spectrum insecticides. This practice, although effective against the target insects, also kills beneficial organisms.

It has also been shown that populations of non-target organisms may fluctuate in conventional cotton fields compared to those of Bt cotton, since the density of a pest may have consequences on the abundance of predators and parasitoids (Romeis et al., 2006). The reduced applications of the broad-spectrum pesticides may favor the increase of beneficial insect populations. However, a lower number of lepidopteran eggs and larvae in Bt cotton can affect the availability of food and hosts of natural enemies. Since the vast majority of these biological control agents have broad diets, the decrease in eggs, and larvae of lepidopteran insects affects their populations only temporarily (Theiling and Croft, 1988; Bradbury and Coats, 1989; Pisa et al., 2015).

Considering the ongoing controversy regarding the environmental impact of Bt cotton and particularly the scarce information on its effects on the diversity of the non-target insects under Mexican conditions, a study was carried out comparing arthropod populations in non-Bt and Bt cotton in the states of Durango and Coahuila (known as "La Laguna;" Nava-Camberos et al., unpublished results). Key target pests *H. zea* and *S, exigua* were only abundant in non Bt-cotton, while no differences were found in overall arthropod species composition and abundance between conventional and Bt-cotton areas. Among them, insects of three orders (Hemiptera, Thysanoptera, and Diptera) and three families (Aleyrodidae, Anthocoridae, and Thripidae) were the most abundant. At the trophic level, the total number of entomophagous and phytophagous insects was similar in both types of cotton. However, the non-Bt cotton presented a reduced diversity index, after several applications of insecticides (Nava-Camberos et al., unpublished results).

Evolution of Resistance in Insects

One of the most important economic risks of genetically modified crops is the evolution of resistance to Cry proteins by insects (Tabashnik et al., 2008) and to herbicides by weeds (Powles, 2008; Heap, 2018). In the case of Bt crops, the evolution of resistance to these crops has already been reported in different parts of the world in Bt corn and Bt cotton that express a single Cry protein (Tabashnik et al., 2008, 2013) or two Cry proteins (Jurat-Fuentes et al., 2003), although there has been no report of such resistance in Mexico (Tamez, 2010; Aguilar-Medel et al., 2017; Mota-Sanchez and Wise, 2018).

One strategy to delay the evolution of resistance is the deployment of "refuges," which consist of plots with non-Bt plants near GM crops (Georghiou and Taylor, 1977; Gould, 1998). For the refuge strategy to be effective, insect resistance should be recessive (Carrière et al., 2010). This means that a resistant insect must carry two copies of the resistant allele. Heterozygous individuals with just one copy of the recessive allele are sensitive to a Cry toxin present in Bt cotton, and only homozygous individuals carrying two copies of the resistant alleles survive on the Bt plants. Therefore, the refuge has the purpose of maintaining a healthy population of susceptible insects. The idea is that when homozygous susceptible insects from the refuge mate with the resistant from the Bt crop field, their progeny will be heterozygous, meaning that they will have one susceptible allele, and one resistant allele. If this occurs effectively in the fields, the pest population will remain sensitive to the Cry toxin expressed in the Bt crop (Andow and Alstad, 1998).

Nevertheless, if two heterozygous insects mate, ¼ of their progeny will be resistant. For this reason it was suggested that in addition to the refuge strategy, the stacking of two or more *cry* genes that have different modes of action has been widely used to delay the evolution of resistance to Bt crops. For example, the stacked MON-88913-8 X MON-15985-7 event expresses the Cry1Ac and Cry2Ab toxins, which have been shown to have a different mode of actions, as they recognize distinct protein receptors in the guts of the same sensitive larvae (Caccia et al., 2010). The consequence that Cry1Ac and Cry2Ab recognize different proteins in the target pests, greatly reduces the probability of having a pest with double mutation (Caccia et al., 2010).

The eradication program of *P. gossypiella* implemented in the United States and Mexico since 2002 established the use of Cry toxins in conjunction with other control strategies. The adoption of Bt cotton with dual toxins by the local farmers resulted in the dramatic decline of this insect and its practically eradication in the Northern region of Mexico (SAGARPA, 2012, 2016; Martínez-Carrillo, 2015).

However, the secondary lepidopteran pest, *S. exigua* shows low susceptibility to Cry1A and Cry2A toxins, and recently it is causing significant damages to the Bt cotton crop in Mexico. To overcome this issue a new stacked event containing the *vip3Aa* gene plus *cry1A*, and *cry2Ab* (Kurtz et al., 2007; Carrière et al., 2015) might be deployed. Vip3A is a highly effective Bt protein that exhibits high toxicity against *S. exigua,* and it has a different mechanism of action than Cry proteins, thus these new pyramided events expressing also Vip3A could effectively control *S. exigua* (Lee et al., 2003; Chakroun et al., 2016). Therefore, this new stacked-Bt cotton variety has a wider spectrum of control than the previous ones, and it will be very helpful in insecticide resistance management. However, due to the high usage of Bt cotton in the American continent, the eventual evolution of resistance, even to the newly stacked events, cannot be ruled out. Therefore, it is necessary to continue searching for novel insecticidal proteins with different modes of action and high efficacy against different cotton pests.

Evolution of Resistance in Weeds

Regarding resistance to herbicides, the first cotton events used in Mexico and elsewhere contained glyphosate resistance genes, which caused an intense use of this herbicide in fields of GM cotton in very large areas of the planet, with the consequence of the evolution of resistance to glyphosate by a diversity of weeds (Powles, 2008). Currently, there are 40

weed species already resistant to glyphosate (Heap, 2018). For this reason, it is recommended the use GM cotton resistant to alternative herbicides with different mechanism of action and other integrated weed management practices that would allow an effective control of weeds, avoiding the evolution of herbicide resistance.

It is interesting that in Mexico there are no reports of weed resistance to the herbicides used in GM cotton (SENASICA, 2016; Heap, 2018). This may be due to the fact that Mexican cotton farmers commonly use conventional tillage and in-row cultivation. Adoption of no-tillage systems in herbicide-resistant GM crops seems to be part of the problem of evolution of herbicide-resistant weeds in countries such as USA, Brazil, and Argentina (Powles, 2008). To cope with this problem there has been a worldwide request to release events that have more than one gene of resistance to different herbicides such as ammonium glufosinate and glyphosate or glyphosate and dicamba.

Thus, in Mexico, the deep tillage along with manual removal of early weeds, in-row cultivation, and crop rotation have apparently delayed the appearance of glyphosate-resistant weeds despite the fact that GM cotton technology has been adopted for more than 15 years (CIBIOGEM, 2018). In contrast, in the United States the first case of Palmer amaranth *A. palmeri* resistant to glyphosate was reported in 2005 (Culpepper et al., 2006), only 8 years after this technology was adopted (Norswhorty et al., 2016).

CONCLUSIONS, RECOMMENDATIONS, AND PERSPECTIVES

G. hirsutum is a native species in Mexico, from which several highly efficient GM cultivars have been developed for the production of cotton worldwide, and some of them are now used in the north region of Mexico.

The tetraploid cotton *G. hirsutum* has a relatively large genome and diverged from its diploid ancestors several million years ago (Shan et al., 2016). Due to the distribution and chromosomal composition of this species, it is expected that there is low risk of introgression or mixing with other diploid wild species of Mexico by pollen flow, but seeds represent an important risk. However, it is still possible that the mixing of GM cotton with wild populations of the same species or another tetraploid specie occurs. It is also possible that the effect of this introgression may be diluted in the wild by processes like meiotic drive or by the lack of selective pressure to maintain the GM genes in complex communities and if the GM genes represent a cost to carry and to express them. Nevertheless, direct experiments will be required to follow the introgressed plants for several generations in the field. Also, given the possibility of introgression is a potential risk, careful monitoring programs for transgenes should be maintained, in particular focusing on the fate and dispersal of the seeds due to spills that occur during transportation from the fields to the gins.

We need detailed socioeconomic studies, as well as epidemiological studies on the health of Mexican cotton farmers, as nowadays there is not enough data to conclude on those aspects.

So far no cases of weed resistance to glyphosate associated with cotton have been reported in Mexico (Heap, 2018). However, it is strongly recommended to encourage the use of appropriate management practices and alternative herbicides with different mechanism of action to delay the evolution of resistance to glyphosate (Devine et al., 1992). In cases of resistance, the use of GM-glyphosate resistant seeds should be avoided since there is a greater danger of increasing the populations of glyphosate-resistant weeds species. This has already occurred in the United States, where weeds such as Palmer amaranth, Johnson grass and barnyard grass are now resistant.

It is known that the use of herbicides with two or more modes of action significantly delays the evolution of herbicide resistance (Neve et al., 2011). Besides, it is necessary to continue integrating the use of herbicides with other management practices, such as deep tillage, in row cultivation, and crop rotation to diversify weed management and decrease selection pressure for herbicide resistance.

The impact of Bt cotton on the use of chemical insecticides has been significant. Since its introduction 20 years ago, there has been a decrease in the use of chemical insecticides, but the data varies between regions due to differences in the ecological and management conditions, different composition of pests and other non-target pests. The evolution of resistance in target-pests cannot be ruled out, even despite the proper use of refuges.

The reduction in the number of applications ranges from one application in Sonora and Mexicali, to almost five applications of chemical insecticide per crop cycle in La Laguna. Also, it is important that the chemical insecticides that are currently used to control the pest complex have, in average, a lower environmental impact than the ones used a couple of decades ago.

Despite the relative good news, it is necessary that farmers and cotton technicians continue to get involved in the detection of a possible loss of efficacy of Bt cotton against the target pests. It is very important also to maintain the active participation of farmers and technicians for the prevention of the evolution of resistance, particularly in the adequate implementation of refuge areas.

In the future, the integration of various pest management tactics will be important, such as cultural control through the destruction of crop residues and biological control through the use of natural enemies (entomopathogens, predators, and parasitoids). The monitoring of insect resistance to Cry toxins expressed by the approved cultivars and those that are envisaged for their introduction in the Mexican market should continue. Federal support for cotton producers is considered crucial to continue with the Binational (Mexico-USA) Program for the eradication of *P. gossypiella* and *A. grandis*, in several regions to declare more free zones in a short term.

The change in the composition of primary insect pests and the increasing possibility of the development of glyphosate-resistant weeds, suggest the urgent need of developing new biotechnological tools to meet national needs. Policies directed toward federal funding for scientific research in Mexico, as well as a national program of seed production should be also strongly encouraged. Mexico has now the human and scientific capabilities and consistent funding of long-term goals directed

to a more sustainable agriculture is needed. This is particularly important due to the lack of possibilities for producers, since there is no national policy for seed production, which puts at risk not only cotton, but also the national food security. Today Mexico depends totally on seeds from the large international companies for its cotton production.

Mexico has been careful in observing the principles of the Cartagena Protocol and the national regulation is highly demanding and expensive to meet. However, in many cases these regulations can only be met by the large companies; as a result, researchers and national institutions with low budgets find impossible to comply with all the requirements established in the biosafety law.

Finally we strongly recommended the agricultural and scientific authorities of Mexico to support a healthy long-term program of national research in order to meet the new needs of agriculture, conventional or GM, for the next 20 years.

ETHICS STATEMENT

Either ethics approval or written consent are not necessary in our study because it is not a clinical study, but instead shows a collection of technical opinions of a group of experts and did not involve disclosure of sensitive personal data. According to the Declaration of Helsinki, medical research is subject to ethical standards that promote and ensure respect for all human subjects and protect their health and rights and it is addressed primarily to physicians. In this work the information obtained from technicians and farmers is related to the crop cultivation practices, and it did not involve any medical study.

AUTHOR CONTRIBUTIONS

MR-M, VS, and AB coordinated the research. LE and MS planned the obtainment of the data. EN, SC, ES collected data and apply surveys. DM-S, ER-R, UN-C, JM-C, CB, MR-M, EN, and SC designed the technician's survey. SC and ES performed the geographical modeling. All authors contributed with the data analyses, discussion of the results, and writing of the paper.

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"Born to Run"? Not Necessarily: Species and Trait Bias in Persistent Free-Living Transgenic Plants

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The possibility of transgenes from engineered plants ending up in unmanaged populations with undesirable consequences has been a long-term biosafety concern. Experience with traditionally improved plants reveals that most cases of such gene escape have been of little consequence, but on occasion they have led to the evolution of problematic plants or have resulted in an increased extinction risk for wild taxa. Three decades have passed since the first environmental release of transgenic plants, and more than two decades since their first commercialization. Examples of transgenes gone astray are increasingly commonplace. Transgenic individuals have been identified in more than a thousand free-living plant populations. Here I review 14 well-documented consolidated "cases" in which transgenes have found their way into free-living plant populations. Some as transient volunteers; others appear to be persistent transgenic populations. The species involved in the latter are not representative of the current commercialized transgenic crops as whole. They tend to share certain traits that are absent or rare in the transgenic crops that do not exist as persistent populations. The traits commonly occurring in species with persistent transgenic free-living populations are the following, in descending order of importance: (1) a history of occurring as non-transgenic free-living plants, (2) fruits fully or partially shattering prior to harvest, (3) have small or otherwise easily dispersed seeds, either spontaneously or by seed spillage along the supply chain from harvest to consumer, (4) ability to disperse viable pollen, especially to a kilometer or more, (5) perennial habit, and (6) the transgene's fitness effects in the recipient environment are beneficial or neutral. Based on these observations, a thought experiment posits which species might be the next to be reported to occur as free-living transgenic populations.

Keywords: dispersal, engineered genes, feral plants, unmanaged populations, pollen gene flow, seed gene flow, seed spillage, volunteers

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INTRODUCTION

An early concern regarding genetically engineered plants was that the unintended movement of transgenes by seed, pollen, or even individuals might have undesirable consequences. The initial focus was that spontaneous hybridization between a transgenic crop and a nearby wild or weedy relative would result in the evolution of a new plant pest (Colwell et al., 1985; National Research Council, 1989). Goodman and Newell (1985) summarized the concern succinctly: "The sexual transfer of genes to a weedy species to create a more persistent weed is probably the greatest

environmental risk of planting a new variety of crop species" noting that the risk is not necessarily restricted to transgenic varieties. Indeed, even though spontaneous hybridization between non-transgenic crops and wild plants is usually of little consequence, in a few cases, such hybridization has had economically disastrous consequences, such as the evolution of Europe's weed beet (Ellstrand, 2003) and Brazil's herbicide-resistant weedy rice (Merotto et al., 2016). Experience from traditionally improved crops has demonstrated that wandering crop genes can have other negative environmental effects. For example, crop-wild hybridization between the domesticated coconut palm and its wild ancestor has resulted in the extinction of the latter (Ellstrand, 2003).

The unintended movement of crop genes was an agronomic problem long before plants were genetically engineered. The primary problems were associated with intervarietal mixing via pollen or seed. Immigrant gene flow by pollen from cross-compatible plants outside of a breeder's selection plots ("pollen contamination") would result in seeds sired by non-experimental plants and frustrate plant improvement efforts. Imagine the offspring from unexpected cross-pollination between backyard pumpkins and a breeder's yellow crooked-neck squash. Consequently, breeders attempt to spatially isolate their experimental plots as well as their seed multiplication fields from possible sources of unwanted pollen (Kelly and George, 1998). Cross-pollination is not the only cause of unintended genetic admixture. Segregation strategies are necessary to prevent accidental mixing of seeds of different commercial varieties ("seed contamination") to maintain varietal purity (identity preservation) for the consumer, Strayer, 2002). To illustrate, a farmer intending to grow sweet corn would be disappointed to find that 20 percent of her plants were a popcorn variety.

With the recognition that 100% genetic purity is difficult or impossible to obtain, acceptable thresholds of unintended genetic material have been standardized for different crops and their purposes. For example, the Organization for Economic Co-operation and Development (OECD) Seed Scheme requires a minimum of 99.7% varietal purity for oilseed groundnut (aka peanut) to be used for basic seed (seed used as the basis for varietal seed increase) and reduces the requirement to 99.5% for certified seed, the purest type of seed normally grown by commercial farmers (OECD, 2018). Generally, farmers and others who deal commercially with crops and crop products anticipate and tolerate low levels of genetic mixing. Given the vigilance of the seed industry to minimize contamination, low levels of within-crop varietal seed admixture have rarely caused substantial harm.

Regulators recognize gene flow in their decision-making and that it is sometimes likely to occur. Therefore, the consideration of transgene flow and its consequences is a standard component of national regulatory risk assessment. For example, in the United States, transgenic plants are allowed to be grown under Notification or Permit only if the applicant describes methods of preventing gene flow (National Research Council, 2002). Likewise, the deregulation in the United States considers the impacts of possible varietal intermixing as well as the establishment of the transgene in free-living populations. Potential gene flow impacts have sometimes led to controversy during the deregulation process in the United States. Such controversy catalyzed the requirement for the United States Department of Agriculture to conduct appropriate studies and create an Environmental Impact Statement for certain regulated articles to be deregulated. Note that such assessments are conducted only for the country involved in the regulatory decision. It is not necessary and perhaps improper, for, say, the United States regulators to make a judgment about the environmental impacts of a transgenic crop in its center of origin (e.g., maize in Mexico) (National Research Council, 2002).

Thus, it is not surprising that unintentional intervarietal mixing by seed or cross-pollination involving transgenic cultivars is not uncommon. Furthermore, the extraordinary sensitivity of polymerase chain reaction–based techniques allows the detection of transgenes at extraordinarily low frequencies (Demeke et al., 2006) The unintended occurrence of transgenes or a transgenic variety is increasingly characterized by the terms "adventitious presence" (Kershen and McHughen, 2005; Demeke et al., 2006; Council for Agricultural Science Technology, 2007), and "low level presence" (Stein and Rodríguez-Cerezo, 2010; Smyth et al., 2017) as alternatives to "contamination," perhaps because the latter carries negative connotations in other contexts. The two new terms are often used interchangeably. However, while adventitious presence of transgenes often occurs at a "low level," but it does not necessarily require that the frequency of the unexpected genetic material be "low."

Reports of transgenes out-of-place have steadily accumulated (Price and Cotter, 2014) since the commercialization of transgenic crops in the mid-1990s. These reports frequently attract the attention of the popular press (e.g., Ledford, 2007). A few scholarly reviews have inventoried the many heterogeneous cases of transgenes in a wide variety of unintended venues (Ellstrand, 2012; Bauer-Panskus et al., 2013; Ryffel, 2014). Those reviews focus on the examples of transgene flow, transgene flow's potential consequences, and improved containment. But none have focused on the biology of the species and traits involved in free-living populations as lessons for environmental biosafety risk assessment.

Initially, the assumption was that all crop transgenes would end up in free-living populations. More than a decade ago, Marvier and Van Acker (2005) stated "the movement of transgenes beyond their intended destinations is a virtual certainty." It is a good times to test that hypothesis. A handful of transgenic crops have been planted in ever increasing acreage for two decades. If "movement beyond intended destinations" is a virtual certainty, that most widely planted transgenes should have all moved beyond their intended destinations by now. The data presented in the earlier reviews suggests that this is not the case.

In particular, to my knowledge, the following questions have not been addressed: Is there anything biologically different about those species and their transgenic traits that set them apart from the species that have not had their transgenes establish

on their own? Have any of the free-living populations created environmental/agronomic problems? Are there any biological correlates for those cases? Given that both genetic engineering and gene editing are likely to soon lead to an accelerating number of products involving of a proliferation of improved species based on an abundance of novel traits, the ability to separate escape-prone trait-species combinations from others should expedite risk assessment in a way similar to the "tiered" approach offered by a recent National Research Council (2017) report.

Here I review the free-living plant populations (volunteer, feral, weedy, and wild) that have been found to have transgenic individuals. I identify which have created environmental (agronomic or otherwise) problems. I focus on the biology of species that have established persistent (multiyear) populations to identify any commonalities. I conclude with a crude model for predicting the kinds of plants with novel traits that might establish free-living populations in the future.

MATERIALS AND METHODS

Incidents of plant transgenes in unintended situations are now too numerous to inventory individually. Thus, I sought and organized "cases" that represent specific combinations of plant species, transgene regulatory status at discovery, and occurrence type. Cases were collected from pre-existing reviews (Ellstrand, 2012; Bauer-Panskus et al., 2013; Ryffel, 2014) and supplemented with a literature review, with special attention to incidents not covered in the previous reviews. Despite that effort, the review is not necessarily exhaustive. Some cases are too poorly studied or documented to report here. Here the concentration is on the most convincing and informative examples. Given the frequent of such reports, I anticipate that new examples will be reported before this article reaches publication. All the cases selected are substantiated by peer-reviewed scholarly articles and/or government publications.

I used the following criteria for choosing the cases:

1) Plants sampled were free-living. That is, sampled plants were growing without intentional human management to promote their survival and growth. Free-living plants can be volunteers, feral individuals, weeds, or wild plants. Unmanaged sites may or may not be human-disturbed. Free-living plants can also be growing within a planted crop. Judging whether or not free-living populations are persistent is often challenging without multi-year study or genetic analysis. Indeed, populations of roadside volunteer plants that are constantly replenished by seed spilled from transport vehicles persist and are considered persistent here. They are analogous to natural wild populations in marginal sites maintained by natural seed rain from nearby robust populations of the same species (Harper, 1977).
2) One or more individuals containing at least one transgene are identified from the population.
3) The transgene is confirmed by more than its gross phenotype. For example, plants surviving a glyphosate application must have a transgene or its product be confirmed by immunochemical or DNA-based analysis.

To maintain a focused scope, the following, somewhat idiosyncratic, categories of transgenes out-of-place were not considered for this review:

(1) Grain or seed mixed with adventitious transgenic grain approved for cultivation in the region grown (e.g., Booker et al., 2014).
(2) Transgenic plants, grain, or seed not at all approved for cultivation that is mistakenly cultivated, but not does not occur in free-living populations (e.g., USDA, 2007, 2017; Clapp, 2008; Dyer et al., 2009; Bashandy and Teeri, 2017).
(3) Grain or seed with transgenes approved for cultivation in the country of origin but unintentionally distributed to countries in which it is not approved but is not known as free-living individuals in such countries. This criterion excludes the notorious "Starlink" affair (see box 2.1 in National Research Council, 2004) and other cases (e.g., University of California Davis, 2003).
(4) Intentionally cultivated transgenic crops unapproved for cultivation in the region grown (that is, intentional illegal cultivation, such as transgenic soy in Brazil prior to its formal approval (Daroit and Nascimento, 2009); and
(5) Spontaneous interspecific or intervarietal seed produced involving a transgenic parent but not resulting in any free-living plants (e.g., Zapiola and Mallory-Smith, 2012).
(6) Coexistence issues (e.g., Devos et al., 2009), that is unintended successful paternal fertilization (gene flow by pollen) by a transgenic crop into a crop grown for a market that is intended to be free of materials from transgenic plants (such as plants grown organically in the United States; Manshardt et al., 2007).
(7) Cases of non-compliance involving "alleged" volunteers (e.g., www.aphis.usda.gov/aphis/ourfocus/biotechnology/sa_compliance_and_inspections/ct_compliance_history).

The foregoing excluded cases are interesting and important in their own right but fall beyond the scope of this review.

RESULTS AND DISCUSSION

Table 1 summarizes 14 cases of transgenes occurring in free-living populations. Altogether, they represent more than a thousand transgenic populations that are the result of dozens of dispersal incidents. The majority of the 14 cases involve multiple plants and populations. One case involves a single transgenic interspecies hybrid individual.

In many cases, the probable dispersal incidents are well-known. In some of those cases, the transgene had entered pre-existing established free-living populations. In others, the transgenic plants themselves appear to be volunteers or the founders of new transgenic populations. And in a few cases, it is difficult to determine their precise origins.

Contrary to initial concerns, crop transgenes have moved into truly wild populations in only a minority of cases. Four entries in **Table 1** detail movement of transgenes into the wild: a herbicide tolerance event from oilseed rape into two populations of wild birdrape in Quebec, Canada (Warwick et al., 2003, 2008), an

TABLE 1 | Free-living[a] populations containing transgenic plants.

Species (common name)	Nature and location of transgenic plants	Transgene: trait[b], regulatory status[c]	Probable process of transgene origin	Current environmental or agronomic impact ?	Relevant citation(s)
Agrostis stolonlifera (creeping bentgrass)	Increasing numbers (hundreds) of glyphosate tolerant plants in the US state of Oregon in ca. 100 sq. km. sampled area. Persistent and spreading.	GT*	Initially, spontaneous seed and pollen dispersal from field test sites in Oregon. Subsequent pollen-mediated gene flow spreads the transgene.	Yes, weed of irrigation canals.	(Zapiola et al., 2008; Zapiola and Mallory-Smith, 2017) and references therein
A. stolonilfera (creeping bentgrass) *x. A. gigantea* (redtop)	Infrequent transgenic hybrid individuals in the US state of Oregon	GT*	Spontaneous hybridization with transgenic *A. stolonilfera* grown under "field trial" conditions as male parent or transgenic free-living plants described in the entry above	No	(Zapiola and Mallory-Smith, 2017)
B. napus (Argentine oilseed rape)	Hundreds of largely persistent populations along grain transport lines in Japan (> 400) and Switzerland (13), both of which prohibit cultivation of transgenic oilseed rape. Multi-year studies show frequency of transgenics varies over time and space	GT^+,gT^+	Repeated seed spillage during grain transport from ports and international borders to processing facilities. Subsequent cross-pollination among varieties gives rise to multiple herbicide tolerance	No.	(Hecht et al., 2014); (Katsuta et al., 2015) and references therein
B. napus (Argentine oilseed rape)	Most plants in several persistent populations in three separate cultivated and non-cultivated areas in Buenos Aires Province, Argentina	GT^+	Unknown, no recent records of oilseed rape production in those locations.	Unknown. (Alternative herbicides are available to control this localized agronomic weed.)	Pandolfo et al., 2016
B. napus (Argentine oilseed rape)	Hundreds of volunteer and persistent feral populations in agronomic and ruderal environments in countries in which transgenic canola is cultivated: Canada (provinces of Alberta, British Columbia, Manitoba, and Saskatchewan), U.S. (states of California and North Dakota), and the Australian state of Western Australia	GT, gT	Spontaneous seed dispersal prior to harvest. Seed spillage during grain transport. Subsequent cross-pollination among varieties gives rise to multiple herbicide tolerance.	Yes. "Volunteer" oilseed rape has become an important agricultural weed of Manitoba and elsewhere in Canada. Evolution of multiple herbicide tolerance through cross-pollination makes control more challenging.	Hall et al., 2000; Yoshimura et al., 2006; Knispel et al., 2008; Knispel and McLachlan, 2010; Schafer et al., 2011; Munier et al, 2012; Busi and Powles, 2016;
B. napus (Argentine oilseed rape) x *B. rapa* (wild birdsrape)	2 transgenic wild x crop hybrid swarms adjacent to cultivated oilseed rape in Québec, Canada. Present for multiple years. When last sampled, transgene frequency decreasing	GT	Spontaneous hybridization with crop as pollen parent.	No.	Warwick et al., 2003, 2008
B. napus (Argentine oilseed rape) x *B. rapa* (wild birdsrape)	Single plant on a Vancouver roadside far from a *B. rapa* population	GT	Unknown	No.	Yoshimura et al., 2006
B. rapa (Polish oilseed rape; domesticated birdsrape)	3 persistent populations in cultivated and disturbed sites in Buenos Aires Province, Argentina; persisting for >4 years	GT^+	Unknown	Unknown. (Alternative herbicides are available to control this localized agronomic weed.)	Pandolfo et al., 2018

(Continued)

TABLE 1 | Continued

Species (common name)	Nature and location of transgenic plants	Transgene: trait[b], regulatory status[c]	Probable process of transgene origin	Current environmental or agronomic impact ?	Relevant citation(s)
Carica papaya (papaya)	22% of feral plants sampled from Oahu and Hawai'i islands of the U.S. state of Hawai'I	VR	Spontaneous gene flow by seed and pollen from cultivated plantations.	No	Manshardt et al., 2016
Gossypium hirsutum (cotton)	Transgenic feral/wild plants identified from multiple sites in each of 4 regions of Mexico. Genetic evidence of multigeneration persistence.	GT,LR	Seed spillage from vehicles carrying post-milled feed grain with some subsequent spontaneous intermating	No	Wegier et al., 2011
Medicago sativa (alfalfa)	Of 404 roadside sites with feral alfalfa plants in the US states of California, Idaho, and Washington, over 100 contained some transgenic plants. Genetic evidence of persistence.	GT	Seed spillage from transports and pollen flow from nearby alfalfa seed and hay production fields with subsequent intermating.	No	Greene et al., 2015
Triticum aestivum (bread wheat)	Modest numbers of plants in a single agricultural field in each of the US states of Oregon (2013), Montana (2014), and Washington (2016). Each incident represents a different transgenic event	GT*	Unknown; each incident seems to be independent of the others.	No. (However, USDA APHIS BRS has changed the field trial application method for transgenic wheat from the notification process to the more stringent permit process)	USDA, 2014, 2015, 2016
Zea mays (maize)	In 2002 and-2004 regulatory inspectors found volunteer transgenic maize engineered to express industrial (including pharmaceutical) compounds growing in soybean, sorghum, and fallow fields in the US states of Nebraska and Iowa	IC*	The volunteers germinated from dormant seed in the soil from the previous year's field trial.	Eradicated.	National Research Council, 2017 (see Box 3.2)
Zea mays (maize)	Extensive sampling over several years finds only modest numbers of transgenic volunteers in the general vicinity of grain-handling ports of South Korea, a country in which cultivation of such plants is prohibited	GT^+, gt^+, LR^+	Seed spillage during grain transport from ports	No	Lee et al., 2009; Park et al., 2009 (Han et al., 2014) and references therein

[a] *"Free-living" signifies populations that occur without intentional human intervention including plants that are volunteers, ferals, weeds, and wild individuals.*
[b] *GT, glyphosate (herbicide)-tolerant; gT, glufosinate (herbicide) tolerant; LR, lepidopteran-resistant; VR; virus resistant; IC expression of industrial compounds*
[c] *No superscript means, "Deregulated in that country or countries at the time of discovery". Otherwise: * not authorized for environmental release globally at time of discovery; + not authorized for environmental release at time and place of discovery.*

herbicide tolerance event from creeping bentgrass field trials into wild populations of the same species and of a congener in Oregon, USA (Zapiola and Mallory-Smith, 2017), as well as and herbicide-tolerant and lepidopteran-resistant events in cultivated cotton into weedy-wild populations of the same species (Wegier et al., 2011).

Seed dispersal, anthropogenic or spontaneous, is a common component of many of those cases. Seed spillage from grain transport appears to have played a major role in the naturalization of transgenic feral Argentine oilseed rape populations (*Brassica napus*) both in countries where it is cultivated and in countries where it is imported but prohibited from cultivation. Seed spillage plays a similar role for feral transgenic alfalfa populations in the United States and feral transgenic cotton populations in Mexico as well as a modest number of volunteer transgenic maize plants near ports in South Korea. In contrast, spontaneous seed and pollen dispersal events from a set of field trials account for the establishment and spread of transgenic creeping bentgrass in the US state of Oregon and beyond.

In a few cases, spontaneous pollen flow alone accounts for the evolution of crop-wild hybrids. Spontaneous pollen flow and subsequent pre-harvest shattering played key roles in the evolution multiple transgenic herbicide tolerant oilseed rape in Canada (Hall et al., 2000). But, for a greater fraction of the cases, the primary role of spontaneous pollen flow in transgene spread

involves cross-pollination among seed dispersed colonists as well as cross-pollination with pre-existing non-transgenic feral plants of that species.

The regulatory status at the time of discovery varies among cases. A substantial minority (5 cases) involve free-living transgenic populations in regions where that crop had been approved for cultivation: alfalfa, Argentine oilseed rape, cotton, and papaya. These situations are of little surprise. The remainder of the cases were equally split between crops whose environmental release were prohibited worldwide at the time of discovery and those whose environmental release was prohibited at the site of discovery but not globally.

Let's return to the hypothesis that "the movement of transgenes beyond their intended destinations is a virtual certainty" (Marvier and Van Acker, 2005) and examine how it holds up when the unintended destinations are free-living populations. A handful of transgenic crop species have been planted in ever increasing acreage for almost two and a half decades: mostly notably maize, soybean, and cotton, but also oilseed rape, papaya, and squash. If "movement beyond intended destinations" is a virtual certainty, those should have all moved by now.

Have they? For certain long-standing transgenic crops, the answer is yes. There are good numbers of free-living populations of papaya, cotton, and (especially) Argentine oilseed rape. But long-standing transgenic soybean has yet to volunteer or go feral (and not without monitoring for its escape; e.g., Lee et al., 2009). Its transgenic partner, maize, has only occurred as first-and-last-generation volunteers in a tiny number of cases. For these two crops the virtual certainty of establishment has not been realized, possibly because they have been handicapped for free-living by their particular history of domestication (Owen, 2005). Another old-timer that has stayed on the farm is transgenic virus resistant squash (*Cucurbita pepo*). Although free-living transgenes have been sought in free-living populations of *C. pepo*, they have not been found (Prendeville et al., 2012).

In contrast, two newcomers have rapidly established feral transgenic populations, one of them more than a decade before deregulation. In the case of transgenic creeping bentgrass, pollen flow and a single localized wind event helped the transgene migrate from a set of field trials and establish in dozens of unmanaged sites. In the case of alfalfa, the feral populations are not far from transgenic alfalfa production areas and seed transport lines. The other relatively recently commercialized transgenic crop, sugar beet, has not been involved with the establishment of free-living populations or even resulted in unwanted volunteers at this time.

Thus, the "virtual certainty" seems more certain for certain species. Let's examine the crops in **Table 1** that have the predilection for itinerant trangenes (excluding those known only as volunteers, maize and wheat): alfalfa, oilseed rape (Argentine and Polish), cotton, creeping bentgrass, and papaya—and compare them to the old-timer transgenic crops that prefer to stay at home. Four of the five wandering crops are multi-year perennials. The other major commercial transgenic crops—maize, soy, squash, sugar beet, are not.

Seed dispersal appears to play the most important role in establishing free-living populations. Spillage from transports creates a regular seed rain on the sides of roads for the easily dispersed fuzzy seeds of cotton and even more so for very small seeds. Oilseed rape's seeds are small, about 200,000 per pound, and thus easily dispersed. That crop is not particularly well-domesticated. Rapeseed fruits can shatter (release) some seeds prior to and during harvest, allowing for the establishment of volunteers in and near the cultivated field. Alfalfa produces seeds of roughly the same size, its legumes shatter as easily as the siliques of rape. Cultivated creeping bentgrass cultivars are even less domesticated. They freely shatter their mature seeds. And those seeds are tiny, about ¼ of the size of alfalfa or rape seed. Feral transgenic papayas, typically on roadsides, may owe their establishment to seed dispersal, by birds or seeds thrown from the window of a speeding automobile (Manshardt et al., 2016). Three of the non-free-living long-term commercial transgenic crops—maize, soy, and squash—have large seeds and do not shatter. The fourth, sugar beet, is harvested before it sets seed.

Overall, outcrossing rate and pollen vector do not seem to play a particularly important role in discriminating among these groups. Both contain mostly outcrossing and mostly selfing species. On the other hand, some idiosyncrasies of mating system may be important: Among the stay-at-homes, soybean is the most highly self-fertilized of major commercialized transgenics, and sugar beet is the only crop that must be harvested prior to flowering. Those features may limit their ability to disperse a suitable number of seeds for colonization. For the other group, it is notable that oilseed rape and creeping bentgrass are known to be able to successful pollinate a mate at a distance in excess of a kilometer (Andersson and de Vicente, 2010).

The "virtual certainty" also seems more certain for certain transgenic traits. The vast majority of free-living populations that have been detected have been subject to very strong selection in favor of the trait based on the transgene. For example, prior to the introduction of virus-resistant papayas to the Hawai'ian Islands, the papaya crop and feral plants were in the process of extirpation there by the onslaught of the fatal papaya ringspot virus (Gonsalves, 2004). A gene for virus resistance would be strongly favored in that sort of environment. In contrast, virus-resistant squash has not established in free-living populations, and the viruses for which it is resistant are now known to typically play a minor role in regulating free-living populations (e.g., Quemada et al., 2008). In those environments, such resistance would confer only a minor advantage, if any (also, see Sasu et al., 2009).

The same logic holds for the abundant free-living populations bearing herbicide tolerance. Herbicide tolerant transgenes are favored in environments in which the selective herbicide is frequently used. We would expect glyphosate tolerance to be especially favored as glyphosate is "the dominant herbicide worldwide" (Duke and Powles, 2008). Indeed, only two of the 14 entries in **Table 1** do not include glyphosate tolerance. Nonetheless, some caveats are appropriate. First, the abundance of herbicide-tolerance in free-living populations may be a simple correlate of the fact that it is, by far, the most abundant transgenic trait among the commercially grown varieties. Also, the trait

is easily detected and noticed when a field is treated with the selective herbicide, revealing the tolerant survivors. Other traits, such as lepidopteran resistance, can only be identified with biochemical tools.

Of the 14 cases of free-living populations, a minority are problematic. Feral populations of multiple herbicide-tolerant transgenic Argentine oilseed rape have contributed to the rise of what is called "volunteer canola" as a significant weed in parts of Canada. Glyphosate tolerant oilseed rape also emerged as a problematic, if local, agronomic weed in the Buenos Aires province of Argentina. Likewise, glyphosate-tolerant creeping bentgrass has become a significant weed of irrigation canals in the US state of Oregon (National Research Council, 2017). The challenges of these problematic weeds are not insurmountable, alternate herbicides can be sought. But that solution is not always straightforward. These weeds have created headaches for farmers who must control them with alternate, less desirable, herbicides (Beckie et al., 2004). In the case of creeping bentgrass, only glyphosate was permitted by the US-Environmental Protection Agency for use as an herbicide in irrigation canals until 2017 when the agency approved a special local label for the use of glufosinate in irrigation canals in Oregon.

What can we learn from these examples about biosafety? With regards to the core principles of biosafety a comparison with traditionally improved plants is illuminating. Gene flow is the "exposure" component of traditional risk assessment's "exposure" x "hazard" = "risk" formulation. We see from the cases in **Table 1** that crop plants already known to feralize or hybridize with free-living populations will do so with or without transgenes in their genomes. But with regard to the realized "hazard" component of the equation, the frequency of problems from free-living populations is somewhat greater than the experience with the feralization of traditionally improved crop plants (Ellstrand, 2003; Ellstrand et al., 2010). That is probably due to the fact that the problem plants have a transgenic phenotype for tolerance to novel herbicides. Notably, the problems are the result of the nature of the transgenic trait and not the result of transgenesis *per se*. Long ago, Ellstrand and Hoffman (1990) wrote, "The ecological impact of crop-weed hybridization will depend more on the biology of the crop, the wild relative, and the transferred gene than on the method of gene transfer." While they did not anticipate the likelihood of direct feralization without hybridization, their emphasis on the biology of the entire system appears accurate.

Perhaps it's now worthwhile to attempt a first draft of a crude model for predicting whether novel alleles (created by any methodology) will establish themselves in free-living plants and, if so, under what circumstances they might contribute to the evolution of increased weediness or invasiveness. As Ellstrand and Hoffman (1990) suggest, let's concentrate on what we know about the biology of the system. Here are some factors that should contribute to the likelihood of novel allele establishment in free-living populations:

1. Crop species that are already known from feral (or wild) populations adjacent to field trials or cultivation. Pre-existing ferality may be a consequence of the following biological traits:
2. Seed dispersal appears to be an important component of the establishment of free-living populations, especially in the case of
    a. Poorly domesticated crops that often shatter or otherwise disperse some of their seeds or fruits prior to harvest
    b. Harvest-to-consumer supply chains that often result in some spillage of seed, grain, or fruit into the environment
    c. The smaller the seed size, the more easily spontaneously dispersed.
3. Spontaneous pollen dispersal appears to play a secondary, but non-trivial, role, especially for those species capable of dispersing viable pollen sufficient distances to wild or otherwise free-living populations. Certainly, crops with the ability of successful pollen dispersal to one kilometer or more should receive close scrutiny, unless it is known that the crop will be fully harvested prior to flowering.
4. Perennial growth occurs at a much higher frequency among the free-living commercial transgenic crops relative to those that have not gone astray. It is not clear (at least to me) why this ecological factor is such a strong correlate.
5. The fitness effects of a transgenic trait as determined by its environment will play a role in its persistence and spread (Ellstrand, 2003):
    a. Detrimental traits are expected to decrease in frequency over time unless replenished by repeated immigrant seed or pollen flow.
    b. Neutral traits are expected to persist at the frequency that they are received.
    c. Beneficial traits are expected to increase in frequency and spread.
    d. Evaluating the selective value of a trait may be challenging. For example, as detailed above, the trait "virus resistance" was beneficial for Hawaiian feral papayas in their specific environment but not for free-living squashes in the United States.

Although determining the fitness effects of a novel trait may be challenging, much of the relevant biological data regarding the crop and its wild and weedy relatives should not difficult to obtain. For example Andersson and de Vicente (2010) book is a good start to evaluate the world's most important crops for details of the seed and pollen dispersal as well as what is known about their feral and wild relatives. Because of the recent research interest on the topic any evaluation should be supplemented with an online literature search. A similar, systematic and structured approach, has been utilized as part of recent environmental risk assessments of upcoming transgenic African crops intended to be grown near related free-living populations (Hokanson et al., 2010, 2016; Huesing et al., 2011).

I finish with a thought-experiment. Which existing transgenic species will be the next to join those in **Table 1**? **Table 2** lists some possible candidates.

All but *Arabidopsis thaliana* have been approved for environmental release in at least one country. *A. thaliana* was chosen for the list because it has been the primary model

TABLE 2 | Potential candidates for as-yet undiscovered free-living transgenic plant populations.

Species (common name)	Transgenic trait(s)
Arabidopsis thaliana (thale cress)	Numerous and varied
Festuca arundinacaea (tall fescue)	Glyphosate tolerance/ Enhanced turfgrass quality
Paspalum notatum (bahiagrass)	ALS-inhibiting herbicide tolerance
Poa pratensis (Kentucky bluegrass)	Glyphosate tolerance/ Enhanced turfgrass quality
Populus nigra (black poplar)[a]	Lepidopteran resistance
Solanum melogena (eggplant)	Lepidopteran resistance
Stenotaphrum secundatum (St. Augustine grass)	Glyphosate tolerance/ Enhanced turfgrass quality

[a]*anecdotal free-living transgenic populations (Bauer-Panskus et al., 2013).*

organism for transgenic research for decades. It has been used as a research organism at hundreds, if not thousands, of colleges, universities, and other research entities. Dozens of field trials have been authorized. Native to the Old World, wild populations have colonized disturbed habitats of pan-temperate regions globally. An adult plant is capable of producing hundreds of tiny seeds (<0.5 mm diameter). The species is largely self-pollinating with opportunity for insect-pollination. The overwhelming global scientific use of this plant suggests that seed spillage might have established volunteer or feral populations of transgenic plants somewhere in the world. If so, they would most likely be in human-disturbed habitats near to where research on the species is conducted.

The rest of **Table 2**'s candidate species have transgenic varieties that are either in cultivation or have been authorized or cultivation. Non-transgenic versions of those species are known to exist in persistent feral or wild populations. All but *Solanum melogena* (brinjal/eggplant) have easily dispersed propagules. *Populus nigra* produces plumed seeds. The rest are grass species with small to very small (especially *Poa pratensis*) caryopses. The grass species are all wind-pollinated but their maximum viable pollen dispersal distances are unknown. *Populus nigra* is dioecious and insect-pollinated. *S. melogena* is largely self-pollinating with opportunity for insect-pollination. All are perennial, and all but *S. melogena* are capable of vigorous vegetative reproduction. Whether their associated transgenic traits confer any fitness advantage depends a lot on the environment in which the free-living populations grow. The abundance of herbicide tolerant cases in **Table 1** suggests that the glyphosate tolerant species in **Table 2** might have an advantage if they disperse into unintended areas in which that herbicide is commonly used. Unless additional confinement features are utilized for these species (e.g., some *Stenophorum secundatum* cultivars are seed sterile), given sufficient cultivation area and sufficient time, taken as a group, it is likely that at least one will donate its transgenes to a free-living population. But, given the foregoing examples, it might take decades for that prediction to be realized.

AUTHOR CONTRIBUTIONS

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

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Transgenic American Chestnuts Do Not Inhibit Germination of Native Seeds or Colonization of Mycorrhizal Fungi

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The American chestnut (*Castanea dentata*) was once an integral part of eastern United States deciduous forests, with many environmental, economic, and social values. This ended with the introduction of an invasive fungal pathogen that wiped out over three billion trees. Transgenic American chestnuts expressing a gene for oxalate oxidase successfully tolerate infections by this blight fungus, but potential non-target environmental effects should be evaluated before new restoration material is released. Two greenhouse bioassays evaluated belowground interactions between transgenic American chestnuts and neighboring organisms found in their native ecosystems. Potential allelopathy was tested by germinating several types of seeds, all native to American chestnut habitats, in the presence of chestnut leaf litter. Germination was not significantly different in terms of number of seeds germinated or total biomass of germinated seedlings in transgenic and non-transgenic leaf litter. Separately, ectomycorrhizal associations were observed on transgenic and non-transgenic American chestnut roots using field soil inoculum. Root tip colonization was consistently high (>90% colonization) on all plants and not significantly different between any tree types. These observations on mycorrhizal fungi complement previous studies performed on older transgenic lines which expressed oxalate oxidase at lower levels. Along with other environmental impact comparisons, these conclusions provide further evidence that transgenic American chestnuts are not functionally different with regard to ecosystem interactions than non-transgenic American chestnuts.

Keywords: risk assessment, allelopathy, ectomycorrhizae, leaf litter, chestnut blight, GMO, restoration

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INTRODUCTION

Transgenic American chestnuts (*Castanea dentata*) have been produced to express an oxalate oxidase enzyme (EC 1.2.3.4), which degrades toxic oxalic acid. Oxalic acid (or oxalate) is produced by the chestnut blight fungus (*Cryphonectria parasitica*) as a virulence factor that kills susceptible American chestnut cambium tissue. Degradation of this acid protects American chestnuts from the lethal effects of blight infections (Zhang B. et al., 2013; Newhouse et al., 2014) without harming the fungus. The oxalate oxidase used in transgenic chestnuts originated from wheat

(Lane et al., 1993), but similar enzymes are found in many other monocots as well as unrelated taxa (Laker et al., 1980; Satyapal, 1993; Molla et al., 2013; NCBI Genbank, 2017), so the enzyme is not foreign to native ecosystems. Before any novel product is used for restoration, however, it is important to evaluate potential non-target environmental effects. This report describes two separate greenhouse bioassays to evaluate potential belowground impacts on other organisms found in American chestnut habitats. Chestnut leaf litter was observed for potential allelopathic effects on germination of native seeds, and chestnut roots were observed for potential effects on colonization by ectomycorrhizal fungi.

Bioassays to observe leaf effects on seed germination have been used to study plants with known allelopathic activity (Lodhi, 1978; Jäderlund et al., 1996; Yang et al., 2016), and to assess insect or microbial interactions with leaf litter from transgenic trees (Vauramo et al., 2006; Seppänen et al., 2007; Axelsson et al., 2011), but we are not aware of other published experiments specifically evaluating effects of transgenic tree leaf litter on seed germination. Given the relatively small number of forest-type deciduous trees transformed to date, it is not surprising that transgenic leaves have not been widely tested for potential effects on wild seed germination. However, assessments of environmental interactions are an important part of potential restoration projects, so such studies are prudent. Non-transgenic American chestnut leaves have previously been evaluated for allelopathic effects, and reduced germination has been reported on some seed species, but negligible effects were reported on other species (Good, 1968; Vandermast et al., 2002).

In nature, fine roots of most plants are colonized by fungi in a mutualistic symbiosis (Smith and Read, 2008). Plants provide their fungal partners with sugar, and fungi provide their plants phosphorus, nitrogen, and other mineral nutrients. It is generally accepted that the vast majority of land plants are normally mycorrhizal, and plants such as American chestnut require these fungi for normal growth. Given the importance of mycorrhizal fungi to American chestnut, it is particularly important to demonstrate that transgenic trees which can tolerate fungal infection above ground will still form partnerships with mutualistic fungi below ground. Transgenic American chestnuts have previously been shown to be no different than wild type Fagaceae with respect to colonization by ectomycorrhizal fungi in laboratory and field bioassays (Tourtellot, 2013; D'Amico et al., 2015). The current study examines newer transgenic lines with higher OxO expression than the 'Darling 4' line used in these older studies. The 'Darling 54' and 'Darling 58' lines used in both current studies express OxO mRNA at levels approximately similar to 'Darling 215' as described by Zhang B. et al. (2013).

MATERIALS AND METHODS

Germination

All chestnut leaves (**Table 1**) were collected from a plot near Syracuse NY in the fall of 2016, dried at room temperature for approximately 6 months, and chopped to approximately 1 cm squares. Sixty grams of each leaf type was thoroughly mixed into 15 L of peat-based commercial potting mix (Fafard Germination Mix, Sungro Horticulture, Agawam, MA, United States), which had been moistened with 2 L of water. This mixture was evenly divided into three standard greenhouse seedling trays (25 cm × 51 cm × 6 cm) with drainage holes.

TABLE 1 | Chestnut leaf types and names used in germination study.

Chestnut type and details	Tree/line name (abbreviation)
Wild-type (non-transgenic) American	'McCabe Hollow' (McCabe)
* Non-transgenic American parent line	'Ellis 1' (Ellis)
* Transgenic American (derived from Ellis parent line)	'Darling 54' (Dar 54)
* Transgenic American (derived from Ellis parent line)	'Darling 58' (Dar 58)
Chinese (*C. mollissima*)	'Qing'
Hybrid (~50% American, ~50% Chinese)	F1 Hybrid (F1)
Third-generation Backcross (Steiner et al., 2017)	B3F3
No-leaf control	No Leaf

Indicates trees of this type were used in the mycorrhizal colonization study.

All seeds were purchased from Sheffield's Seed Company (Locke, NY, United States) in the spring of 2017, and had been cold stratified by the supplier. Seed species were selected to represent different plant types of native species that are found in the traditional range and habitat of the American chestnut: *Elymus virginicus* = grass, *Cichorium intybus* = forb, *Gaultheria procumbens* = shrub, *Pinus strobus* = coniferous tree, *Acer rubrum* = deciduous tree. Twenty seeds of each species for each tray were started on March 21, 2017, either soaked in distilled water overnight (if suggested by the supplier for that species) or placed directly in the moist soil with leaves. Three seed types (*Tsuga canadensis, Cornus alterniflora, Tussilago farfara*, n = 10–15 seeds each) were sown in all trays but did not germinate appreciably in any tray type, so they were not included in subsequent analyses.

Transparent covers 7 cm tall were placed over all trays for the duration of the experiment, except during watering and observations. Trays were kept in a greenhouse at 20–22°C, with supplemental lighting on a 16-h light/8-h dark cycle. Trays were watered weekly; individual trays were watered more frequently if they were observed to be dry. Trays were arranged in three replicated blocks along a long table in the greenhouse, and left in place for the duration of the study.

Germination observations, conducted twice weekly, consisted of counting the total number of seeds that had germinated of each type in each tray (**Figure 1**). At the conclusion of observations for a given species, all germinated seedlings were removed from the tray, tapped and brushed gently to remove loose potting mix, dried at 60°C in a paper bag for 48 h, and total seedling dry biomass was recorded for each species in each tray. (This was conducted at ~4 weeks for *Cichorium*, of which essentially all seeds had already germinated and many were crowding seedlings in adjacent rows, and 8–10 weeks for remaining species.) Mean counts and masses from each tray were analyzed with one-way ANOVA (GLM Procedure, SAS v9.2, SAS Institute, Cary, NC, United States) and compared using Tukey's Studentized Range (HSD) test ($\alpha = 0.05$).

FIGURE 1 | Representative tray to demonstrate germination bioassay in progress. This tray contained a single leaf type, and was one of three replicated trays with this leaf type. Note that *Cichorium* (second row from right) germinated notably earlier than other species, and was accordingly removed earlier to prevent crowding of adjacent rows. The smaller photo at right shows *Cichorium* roots growing through a chestnut leaf piece, demonstrating plant roots clearly interacting with leaf litter in this bioassay.

Mycorrhizal Colonization

Soil samples for mycorrhizal inoculum were collected from the same plot as the leaves, in mixed hardwood forest with sugar maple (*Acer saccharum*), American beech (*Fagus grandifolia*) and Eastern hemlock (*Tsuga canadensis*). Twenty-three samples were taken using a cylindrical soil core 4 cm diameter driven to a depth of 15 cm. The location of each sample was randomly determined. Soil samples were dried, then sifted through a 0.5 cm mesh (USA Standard Soil Sieve). The soil inoculant was mixed at a ratio of 1:1:2 dried soil: sand: sphagnum peat moss. The resulting inoculant mix was split evenly among 45 pots (D40 Deepots, Stuewe & Sons), which had been previously sterilized overnight in a 7% bleach solution. Tissue culture-generated *C. dentata* approximately 6 months old were transplanted into pots containing the inoculant. Three types of *C. dentata* were used: 15 individuals each of 'Ellis 1,' 'Darling 54,' and 'Darling 58' (**Table 1**). These were grown a greenhouse at 21–26°C, with a 16-h light/8-h dark cycle, and watered as needed. The plants did not receive fertilizer or pH amendments during the experiment to encourage associations with mycorrhizal fungi.

Mycorrhizal colonization rate was assessed after 5 months of growth by collecting a continuous root length of at least 15 cm from each surviving plant. All root tips along the sample were observed, and the percentage of root tips with evidence of a fungal mantle, and those without a mantle, were visually estimated using a dissecting microscope and assigned to categorical percentage ranks (e.g., 90–95 or >95%). A root tip was considered ectomycorrhizal if it was actively colonized or senescent with indications that it had been previously colonized. Ectomycorrhizal roots are produced when a mycorrhizal fungus forms a mantle around root tips. Evidence of colonization is readily apparent on chestnut roots (**Figure 2**); fungal mantles are distinctly unique in terms of color, texture, and thickness compared to un-colonized areas of roots. Non-mycorrhizal roots were identified by the lack of a mantle and presence of root hairs. The frequencies of each category in each treatment were calculated. A Fisher's exact test of independence with a significance of 0.05 was used to test the null hypothesis that there was no significant difference in root tip colonization between any two treatments.

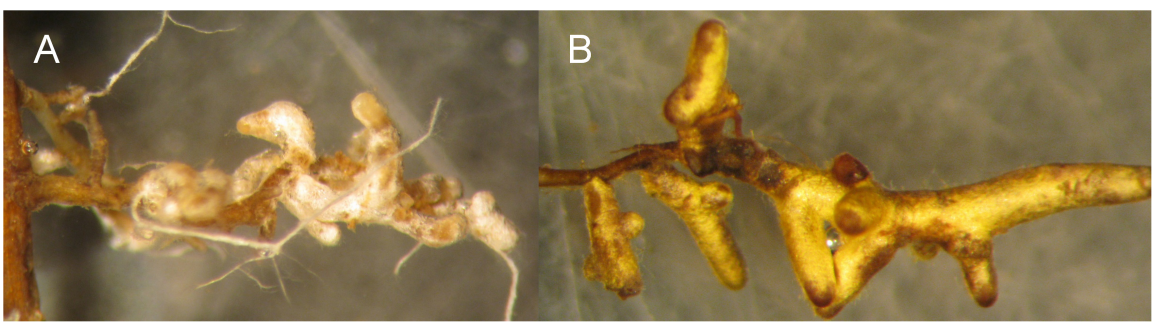

FIGURE 2 | Ectomycorrhizal (EM) root tips harvested from American chestnut seedlings. Plant roots are seen on the left side of both figures as a brownish main (secondary) root. EM roots branch off of these secondary roots and are wrapped in a fungal sheath or mantle. The fungal mantle gives the EM root tips their distinctive colors, with a white morphological type (morphotype) in **(A)** and a golden morphotype in **(B)**. Note the hyphal cords in **(A)**: these are aggregations of hyphae that facilitate transport of sugars from the plant to the fungus in the soil and soil nutrients from the fungus to the plant roots.

RESULTS

Germination

Mean counts and masses of germinated seedlings in all leaf types are shown in **Figure 3**; all mean values with standard error are available as **Supplementary Material**. Tukey's HSD test indicated only a few pairwise comparisons with statistically significant ($p < 0.05$) differences (presented here as mean ± 1 SE). Count of *Pinus* seedlings was significantly different between 'McCabe Hollow' (7.0 ± 1.2 germinants) and the no-leaf control (17.0 ± 2.5 germinants), and the mean biomass of *Cichorium* seedlings was significantly different between 'Darling 58' (1.52 ± 0.19 g) and B3F3 (0.83 ± 0.14 g). There were no significant differences between either of the transgenic leaf types and the non-transgenic 'Ellis 1' control, which is genetically identical to the 'Darling' lines in this experiment other than transgene presence. Allelopathy by chestnut leaves in general was not broadly apparent, as no-leaf control trays showed overall similar germination of most seed species.

Mycorrhizal Colonization

Surviving trees observed in the mycorrhizal colonization study included 15 'Ellis,' 10 'Darling 54,' and 12 'Darling 58.' Mycorrhizal colonization was consistently high among all types, with all but one observed plant showing greater than 95% ectomycorrhizal colonization. The observed root from one 'Darling 54' tree showed 90–95% colonization. According to Fisher's exact tests, there were no significant differences in colonization between 'Ellis' and the transgenic lines 'Darling 54' or 'Darling 58' ($p > 0.40$).

DISCUSSION

The few statistically significant differences in seedling germination between leaf types (*Pinus* counts, *Cichorium* mass) did not represent trends between transgenic and non-transgenic American chestnuts. In both of those contrasts, similar leaf types (**Table 1**) did not show the same patterns. In other words, while the *Pinus* germination count was low on 'McCabe Hollow' leaves, it was not significantly different among other American chestnut leaf types. And while *Cichorium* mass was higher on 'Darling 58' leaves than B3F3 leaves, this difference was not statistically significant compared to American chestnut controls or other leaf types. These differences may therefore be due to individual genotypic differences or random variability, and are within the scope of differences found in other non-transgenic controls in this study. The general lack of germination observed in the three excluded seed species (*Tsuga canadensis, Cornus alterniflora, Tussilago farfara*) may indicate inadequate cold stratification, but its consistency among all leaf types and no-leaf controls suggests it is not the result of leaf interactions or allelopathy.

Previous studies on transgenic leaf litter have more commonly focused on microbial communities or leaf decomposition rates rather than germination of neighboring plant seeds. Not surprisingly, some transgene products in such studies have been observed to produce their intended effect when present in leaf litter: Bt toxins in leaf litter can affect aquatic insect communities (Axelsson et al., 2011) and increased tannin levels in leaf litter can affect moss proliferation and certain microbial classes (Winder et al., 2013). In both of these cases, the changes observed are expected based on the transgene product, and other microbial communities were not affected. The oxalate oxidase enzyme in 'Darling' transgenic American chestnuts is not a toxin and is not known to have allelopathic properties [it actually degrades toxic oxalate which likely *does* have allelopathic properties (Itani et al., 1999)], so the germination similarities among chestnut leaf types are not surprising.

Colonization by mycorrhizal fungi is often one of the first concerns people express when they hear about a wild, non-agricultural plant engineered to tolerate infections by a

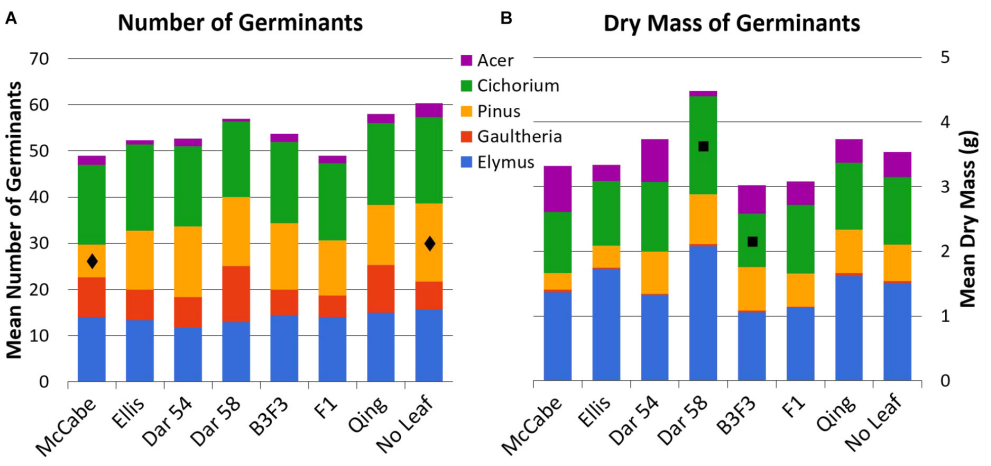

FIGURE 3 | Total mean counts (**A**, $n = 20$ seeds/type) and dry masses **(B)** of germinated seeds for all leaf and seed types. All mean values with standard error are available as **Supplementary Material**. See **Table 1** for leaf types and abbreviations. Statistically significant differences for pairwise comparisons within seed species are noted with ♦ (*Pinus* counts, McCabe vs. No Leaf) and ■ (*Cichorium* mass, 'Darling 58' vs. B3F3).

pathogenic fungus. Such concerns are of course legitimate, especially in cases where broad-spectrum fungicidal traits might be employed. But in the case of 'Darling' chestnuts, the transgene product is not fungicidal in nature, and did not affect mycorrhizal colonization. This study specifically supports previous investigations on the mycorrhizal condition of transgenic American chestnut based on field and laboratory bioassays, all of which indicate no differences in colonization between transgenic and non-transgenic American chestnut roots (Tourtellot, 2013; Dulmer et al., 2014; D'Amico et al., 2015). Even with the higher transgene expression in 'Darling 54' and 'Darling 58' compared to the 'Darling 4' tested previously, there were no significant differences in colonization by ectomycorrhizal fungi in roots compared to non-transgenic controls. These results corroborate other studies that generally show no significant differences between transgenic and non-transgenic plants with respect to colonization of mycorrhizal fungi (Kaldorf et al., 2002; Newhouse et al., 2007; Cheeke et al., 2015; Turrini et al., 2015; Chen et al., 2016; Kaur et al., 2017).

Instead of acting directly on the pathogen, the transgene product (oxalate oxidase) in 'Darling 54' and 'Darling 58' chestnuts protects the host by degrading a toxin produced by the chestnut blight fungus. The toxin, oxalic acid, is associated with wood decay in some fungal species (Clausen and Green, 2003; Hastrup et al., 2012), and in chestnut blight, it is a virulence factor specifically associated with the pathogenic lifestyle of *C. parasitica* (Rigling and Prospero, 2018). In contrast, mycorrhizal fungi depend on the mutual flow of materials between themselves and their plant hosts, and thus have no need for the action of oxalic acid, so its degradation should have no effect on mycorrhizal colonization. Beyond the transgene product itself, the by-products from oxalate oxidase degradation of oxalic acid are hydrogen peroxide and carbon dioxide (Lane et al., 1993). Hydrogen peroxide has fungicidal properties at sufficiently high concentrations (Baldry, 1983; El-Gazzar and Marth, 1988), but in some cases mycorrhizal fungi may actually employ hydrogen peroxide as a control mechanism (Salzer et al., 1999) or signal molecule (Zhang R. et al., 2013). Furthermore, chestnut blight does not typically infect tree roots (Hepting, 1974; Weidlich, 1978), so it is unlikely that substantial oxalic acid degradation (and thus hydrogen peroxide formation) would take place in the rhizosphere.

Collectively, these studies reinforce previous and concurrent findings that transgenic American chestnuts are not ecologically different than non-transgenic American chestnuts (apart from their enhanced blight tolerance). Along with the previous mycorrhizal experiments referenced above, collaborators have preliminarily evaluated aquatic and terrestrial insect feeding on transgenic chestnut leaves and natural introgression of plants near field-planted transgenic chestnut trees (unpublished). Additionally, Gray (2015) and Gray and Briggs (2015) compared transgenic and non-transgenic chestnut leaf decomposition, and Goldspiel et al. (in press) tested wood frog tadpole growth and survival with transgenic chestnut leaves. In each of these cases, transgenic chestnuts showed negligible differences compared to non-transgenic American chestnuts, or smaller differences than traditionally-bred hybrid or Chinese chestnuts. Restoration of wild species such as American chestnut should be approached with care and wisdom regardless of what methods are used to produce restoration material, and interactions with neighboring species are an important part of this biosafety evaluation process.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

AN wrote the majority of the manuscript and helped with setup, observations, and analysis of the germination study. AO helped with design and setup of the germination study and performed statistical analyses for this experiment. HP helped with setup and performed the majority of observations for the germination study. HR helped with setup and performed all observations and statistical analysis for the mycorrhizae study. TH contributed advice on the mycorrhizal experiment and helped with the writing of this part of the manuscript. WP was the chestnut project director and provided lab space, greenhouse space, transgenic plant material for both studies, and valuable support and experimental guidance. All authors reviewed and approved the manuscript prior to submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01046/full#supplementary-material

DATA SHEET S1 | Mean seedling counts and dry biomass measurements (both including standard error) for germinated seedlings with all leaf types.

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Phenotypic Expression and Stability in a Large-Scale Field Study of Genetically Engineered Poplars Containing Sexual Containment Transgenes

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Genetic engineering (GE) has the potential to help meet demand for forest products and ecological services. However, high research and development costs, market restrictions, and regulatory obstacles to performing field tests have severely limited the extent and duration of field research. There is a notable paucity of field studies of flowering GE trees due to the time frame required and regulatory constraints. Here we summarize our findings from field testing over 3,300 GE poplar trees and 948 transformation events in a single, 3.6 hectare field trial for seven growing seasons; this trial appears to be the largest field-based scientific study of GE forest trees in the world. The goal was to assess a diversity of approaches for obtaining bisexual sterility by modifying RNA expression or protein function of floral regulatory genes, including *LEAFY, AGAMOUS, APETALA1, SHORT VEGETATIVE PHASE*, and *FLOWERING LOCUS T*. Two female and one male clone were transformed with up to 23 different genetic constructs designed to obtain sterile flowers or delay onset of flowering. To prevent gene flow by pollen and facilitate regulatory approval, the test genotypes chosen were incompatible with native poplars in the area. We monitored tree survival, growth, floral onset, floral abundance, pollen production, seed formation and seed viability. Tree survival was above 95%, and variation in site conditions generally had a larger impact on vegetative performance and onset of flowering than did genetic constructs. Floral traits, when modified, were stable over three to five flowering seasons, and we successfully identified RNAi or overexpression constructs that either postponed floral onset or led to sterile flowers. There was an absence of detectable somaclonal variation; no trees were identified that showed vegetative or floral modifications that did not appear to be related to the transgene added. Surveys for seedling and sucker establishment both within and around the plantation identified small numbers of vegetative shoots (root sprouts) but no seedlings, indicative of a lack of establishment of trees via seeds in the area. Overall, this long term study showed that GE containment traits can be obtained which are effective, stable, and not associated with vegetative abnormalities or somaclonal variation.

Keywords: RNAi, *Populus*, dominant negative mutations, gene flow, biosafety

INTRODUCTION

Trees provide humans with a variety of useful products, including wood, fiber, energy, and food. In addition to these tangible products, trees also provide ecological services, such as carbon capture, water purification, and by serving as keystone species that promote biodiversity. Plantation ecosystems, though generally less diverse than wild ones, can also promote biodiversity and help to reduce pressure on native forests (Barlow et al., 2007; Brockerhoff et al., 2008).

Genetic improvement is extremely important to orchard and plantation management. Although a wide variety of biotechnologies are used for tree breeding, genetic engineering is of great interest because it bypasses the long generation cycle and intolerance to inbreeding of trees, and allows traits to be added or modified without significant background changes to commercially valuable clones. Examples of genetically engineered (GE) trees include agricultural species such as *Carica papaya* (papaya) (Fitch et al., 1993) and *Malus domestica* (apple) (Boresjza-Wysocka et al., 1999; Murata et al., 2000), forestry species including *Populus* (poplars) (Meilan et al., 2002; Klocko et al., 2014; Yang et al., 2015; Ault et al., 2016), *Eucalyptus* species (eucalypts) (Harcourt et al., 2000; Matsunaga et al., 2012), and even wild and ornamental trees such as *Castanea dentata* (American chestnut) and *Ulmus americana* (American elm) (Newhouse et al., 2007; Sherif et al., 2016) (Maynard et al., 2009; Zhang et al., 2013). However, most of these varieties, exceptions being deregulated virus resistant papaya and non-browning Arctic apple, are not grown commercially (Strating, 1996; Waltz, 2015). This limited uptake by growers and consumers is not due to a lack of success of the traits of interest, but rather due to the controversy surrounding the GE process used to produce them.

A major concern for GE trees is gene flow; the spread of trees or their gametes beyond the boundaries of plantings. Similar concerns about gene flow apply for exotic tree species, which have become invasive in a number of instances (Richardson and Rejmanek, 2011) and could thus benefit from the same containment technologies as discussed for GE trees. Unlike many crops, most trees are perennial, long-lived, and weakly-domesticated—exacerbating gene flow concerns. Gene flow can occur through localized vegetative spread in some species, such as by shoots from spreading roots, and by rooting of detached branches, such as in various species of poplars. In most tree species, however, long-distance spread occurs mostly via sexual reproduction through the movement of pollen or seeds.

Studies of GE tree species have shown that gene flow can and does occur, and its extent varies widely among species and environments. For example, poplar is a wind-pollinated, outcrossing species with potential for long distance spread by pollen and its cottony seeds. Models for predicted gene flow in poplar show that fertility is a key factor for influencing spread, as is the fitness effect of the trait encoded by a transgene (DiFazio et al., 2012). A recent study of insect resistant cry1Ac poplar in China quantified the amount of gene flow between male cry1Ac trees and female trees in the surrounding plantations. They found that the rate of GE seed formation varied from 0.00 to 0.16% of seeds, and no GE seeds were found at distances greater than 500 m from the male trees (Hu et al., 2017). In addition, they also found that seeds purposefully planted in the field failed to germinate unless they received purposeful intervention, such as irrigation, indicating a low risk of seedling establishment. Other studies of transgene flow are from fruit tree species. GE plum pox resistant trees have been developed and are deregulated, but are not in commercial production (Ravelonandro et al., 1997; USDA, 2015). Plum flowers have bee-mediated pollen transfer, and a low rate of gene flow from GE trees (up to 0.215–0.117% of tested embryos), which drops off with distance (Scorza et al., 2013). Even fruit trees that are obligate outcrossers, such as apple, have distance-limited movement of pollen by bees. One study found that at distances of greater than 146 meters, no GE seeds were detected (Tyson et al., 2011). One of the few commercialized GE trees is papaya (Gonsalves, 2006). Field evaluation of pollen flow between GE and conventional stands showed a very low rate of pollen transfer, between 0.3 and 1.3% of embryos tested (Gonsalves et al., 2012). While papaya is wind-pollinated, the varieties grown were bisexual, and were likely self-pollinating. For the fruit trees species detailed above, only pollen-mediated transgene dispersal was studied. The fruits produced by these species are large and fleshy, and may or may not undergo long-distance dispersal in field conditions, depending on the species and nature of foraging by animal dispersers (e.g., birds vs. mammals).

There is a paucity of field data for GE forest trees, and much of it comes from short term trials (reviewed in Strauss et al., 2017). Desired data include assessment of measured ecological impacts of GE trees as compared to non-GE tress, GE tree performance such as growth and survival, and the effects of the specific engineered traits on commercial properties. While laboratory and greenhouse trials are useful for initial assessments, it is known that these results rarely match those obtained in the field. For example, a field and greenhouse test of reduced lignin GE poplar trees found that tree form, size, and wood characteristics differed dramatically between greenhouse and field conditions (Voelker et al., 2011). Similar results have been reported in other studies (e.g., Viswanath et al., 2012). Unfortunately, permits for field trials are often difficult to obtain, in part due to the risk of gene flow into feral and wild populations. Unless flowering is explicitly allowed by permits, trees must be terminated before reaching maturity. However, juvenile trees are known to differ in trait expression, such as for wood characteristics from adult trees (Zobel and Sprague, 1998). Thus, in addition to enabling commercial use, a containment system could have large benefits for enabling field research.

There are several possible means to limit gene flow from trees. Non-GE methods include harvesting prior to maturity, growing varieties that cannot interbreed with nearby populations, creating wide hybrids which are sterile or have limited fertility, seeking and growing rare non-flowering individuals, and using random mutagenesis followed by screening to obtain sterile individuals (Ranney, 2004). Alternatively, genetic engineering can be used to specifically target one or more genes with predicted roles in flowering and/or floral fertility (Vining et al., 2012). Tree sterility could serve as an enabling technology for research and commercial use of trees modified for high-value traits.

This manuscript summarizes the findings from a large-scale field test of GE poplars that were modified with the goal of genetic containment. We report that several methods for direct modification of floral gene expression provide powerful and reliable means for impairing fertility, and thus for preventing or mitigating gene flow.

RESULTS

Regulation and Site Management

The field trial was established in the summer of 2011 as a test of genetic constructs designed to delay or modify poplar flowering for genetic containment. In addition to genetic insights about construct effects, the experience of growing and obtaining regulatory approval for this flowering trial may be of broader interest for biosafety and field studies of GE trees. Regulatory compliance required a large amount of work before the science could even begin. All field tests of GE plants in the US require a permit from USDA APHIS prior to establishment of the plants in field. The work and costs associated with obtaining and meeting the conditions of such permits are significant barriers to field testing. In addition to costs associated with the actual scientific study of the trees, we have paid from our research budgets most of the costs of site preparation, fence maintenance, tree removal, and site monitoring after trial termination. In addition, because flowering and sexual reproduction were key traits under study, the permit had several additional monitoring requirements. The entire site was enclosed in fencing (higher than 3 m) to exclude large herbivores, mainly *Odocoileus virginianus* (whitetail deer). This fence and the gates also served as a deterrent to unauthorized humans, as did the somewhat remote site location (in an agricultural area about one mile from a town). Vandalism by humans at various GE tree locations (lab or field) is a known risk, and did occur at this and one other Oregon State University (OSU) field site in 2001 (**Figure 1**; Kaiser, 2001). Thankfully, human vandalism at this current site did not occur during the duration of this study. Trees that were vandalized by attempted girdling in previous trials were either removed as the trial was scheduled to be terminated (**Figure 1A**), or continued to grow as poplar has the ability to regrow even with removal of bark (**Figure 1B**). A more common source of damage is from herbivores, such as small rodents (**Figure 1C**), and they require constant monitoring and often trapping or toxic methods to manage them when populations are high. Trees can recover from small amounts of herbivore damage; more extensive herbivory can lead to the need for tree replacement. In one case an entire planting was destroyed during its first growing season due to an outbreak of voles at a field site; it was replanted the following year when vole populations crashed (Elias et al., 2012) Other management challenges undertaken by our research team included set up of irrigation, irrigation management and monitoring, irrigation pump repair and maintenance, and repeated weed control during the growing season.

In addition to routine management, regulatory requirements stipulate the need for frequent, documented monitoring of the site for vegetative sprouts and unanticipated tree phenotypes (the latter requires a rapid report to USDA). While this trial did not yield any unexpected traits, other trials in the same tract of land have given rise to unexpected traits. For example, a previous field trial testing GE hybrid poplar with modified gibberellic acid signaling (leading to semi-dwarfism) flowered in summer rather than in February, which is very atypical for poplar. A report of this to USDA led to immediate removal of all flowers, though the risk of pollination at that time of year was nil (Strauss et al., 2016). Other unexpected outcomes from previous trials were rare somaclonal variants (Ault et al., 2016; Strauss et al., 2016). No such variants were observed in the current trial. In addition, unanticipated environmental occurrences at the field site must be reported to USDA; in more than one instance a portion of the field site was flooded during heavy winter rains; however, no trees were lost, nor were any flowering at the times.

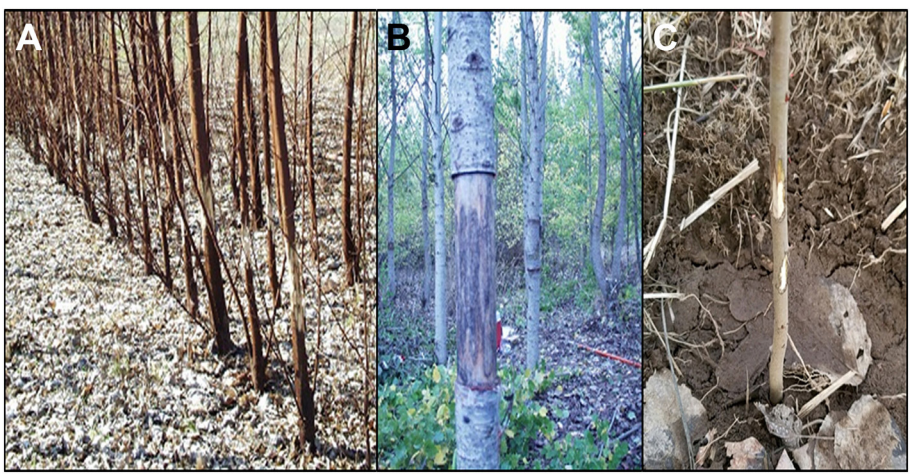


FIGURE 1 | Plantation damage by human vandals and other animals can be problematic. Plantations of **(A)** young and **(B)** mature poplar trees were vandalized by humans "eco"-vandals peeling off bark in 2001. **(C)** In 2017 rodents chewed bark off of young trees.

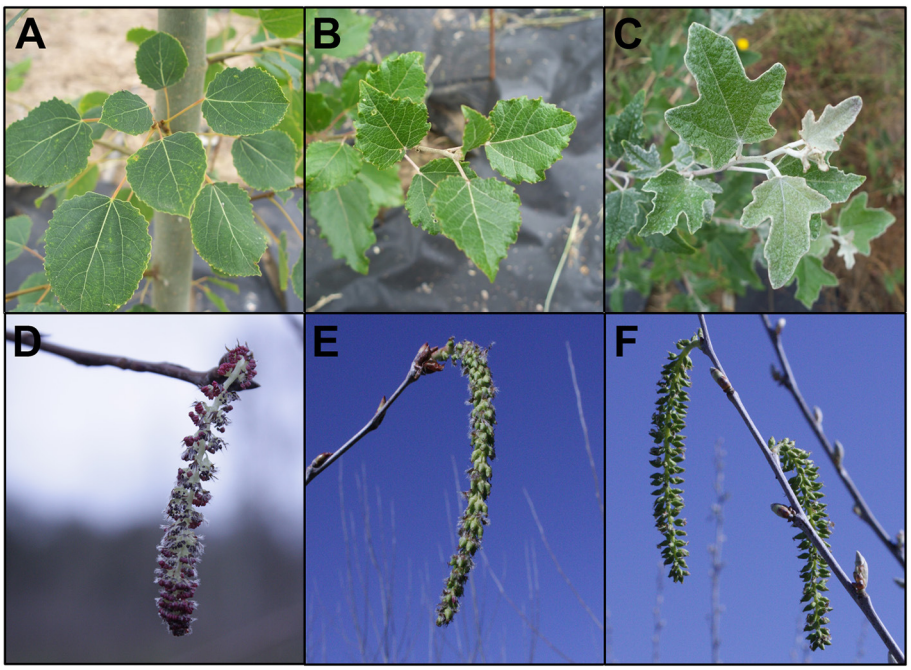


FIGURE 2 | Features of the three clones of hybrid poplar studied. Male clone 353 *Populus tremula x tremuloides* had **(A)** heart-shaped leaves with fine serrations, and **(D)** staminate flowers with red anthers. Female clone 717 *Populus tremula x alba* had **(B)** triangular leaves with larger serrations and **(E)** pistilate flowers. Female clone 6K10 *Populus alba* had **(C)** lobed leaves with a tomentous abaxial surface giving a silvery appearance, and **(F)** pistilate flowers. Foliage images are from July 17, 2012, 1 year after field establishment. Flowers are from the first year of floral opening in the field. Male clone 353 flowers are from February 25, 2015, female clone 717 and female clone 6K10 flowers are from March 21, 2014.

SCIENTIFIC GOALS AND METHODS

While male sterility may be sufficient for containment of some species of plants, many trees (including poplars) have wind-dispersed seeds that can move long distances. Therefore, efficient genetic containment would require a method and gene targets that lead to bisexual sterility. Though most individual trees are unisexual, it is not uncommon to find mixed gender flowers on single trees, even if individual trees are unisexual. Because poplar is predominantly dioecious, we used male and female clones to test effects in both genders. We also used a female clone that flowers early, to speed the ability to obtain results (**Figure 2**). Male clone 353-53 was a hybrid, *Populus tremula x tremuloides,* and had round leaves and staminate flowers with prominent red anthers. Female clone 717-1B4 was a hybrid, *Populus tremula x alba,* and had blade shaped leaves with small serrations and pistilate flowers. Both of these clones were created by scientists at INRA in France. Female clone 6K10 was *Populus alba*, with silvery leaves and pistilate flowers, and rapid onset of flowering; it was identified by the Italian scientist Maurizio Sabatti of Tuscia University, as reviewed in Meilan et al. (2004).

Fifteen different poplar genes were selected as targets or tools for genetic containment (**Table 1**). At the time of vector construction, with the exception of *LEAFY* and its poplar ortholog (Weigel and Nilsson, 1995; Rottmann et al., 2000), none of the genes had been characterized in transgenic poplar and sequence data was limited to cDNAs and the initial release of the *P. trichocarpa* genome sequence. Hence, the genes were selected primarily based on knowledge of, and homology to, genes characterized in *A. thaliana*. Given the paucity of functional data about the poplar gene homologs, we selected genes from different stages in the floral pathway—from signal integration through to determination of floral organ identity—in hope of generating diverse types of sterility, some of which at least would be robust and not impart negative effects on vegetative development. In general, if there were two putative co-orthologs of an *A. thaliana* gene (as is common in poplar; e.g., *AG*, *API*, *FT*), we generated RNAi constructs that were predicted to target both paralogs. Twenty three constructs were designed to target these genes, either singly or in combination (**Table 2**). Some constructs were designed to modify the timing of floral onset or the floral abundance, while others were designed to modify floral organ identity such that anthers or carpels would instead develop as non-reproductive floral organs (**Table 2**). Several constructs targeted two or more different floral development genes.

Constructs were transformed into the three poplar clones and independent transformation events obtained. Vegetative propagation methods were used to obtain an average of four ramets (trees) from each transformation event. Events were planted in two-tree plots to make it easier to visually detect modifications to flowering and vegetative development. Each row-plot was planted at random in each of two blocks for the three poplar clones (they were separated into blocks due to their distinct rates of growth, and thus likely shade induced

TABLE 1 | Genes targeted for suppression or modified expression in transgenic poplar trees.

Gene name(s)	Location(s) in floral pathway	Poplar gene(s) from phytozome	Construct type(s)
FPF1 (FPFL1, FPFL2)	Input from GA pathway	Potri.006G276100, Potri.018G005200, Potri.010G024500, Potri.008G209300	RNAi
AGL20 (SOC1)	Signal integration	Potri.014G074200	RNAi
FT (FT1, FT2)	Signal integration	Potri.010G179700, Potri.008077700	RNAi
AGL24	Signal integration Meristem determination	Potri.002G105600	OvExp, RNAi
LFY	Meristem determination	Potri.015G106900	RNAi
SVP	Meristem determination	Potri.007G010800	OvExp
AP1 (AP1-1, AP1-2)	Meristem determination Floral organ determination	Potri.008G098500, Potri.010G154100	DNM, RNAi
AP3	Floral organ determination	Potri.005G118000	RNAi
AG (AG-1, AG-2)	Floral organ determination	Potri.004G064300, Potri.011G075800	DNM, RNAi

Thirteen poplar genes were selected for suppression or modification in hybrid poplar, both singly and in combination. OvExp, over expression; DNM, dominant negative mutation; RNAi, RNA interference. Genes were selected based on sequences of the initial poplar genome, gene IDs shown are from Populus trichocarpa v3.0.

mortality prior to flowering) (**Figure 3**). A total of 3,315 trees (**Table 3**), including controls, were planted in approximately over 3.6 hectares. These included 1,112 trees of male clone 353, 1,254 trees of clone female 717, and 1,139 tree of female clone 6K10. The plantation was located in Western Oregon, a region characterized by a warm dry summer and cool wet winter. The trees were not protected from the elements and experienced a very hard freeze in 2014 and a usually hot dry summer in 2016. Tree survival was scored yearly; by the end of 2017 survival for all trees was 94.6% (as determined by number of trees currently alive versus number of trees planted). Male clone 353 had the lowest survival of 91.0%, female clone 717 had a survival rate of 94.1%, and female clone 6K10 the highest rate of survival at 98.6%.

Tree size was measured yearly for all trees in the plantation. Both trunk diameter at breast height (DBH) and overall tree height were measured until 2016 (when many trees outgrew the height pole); from 2016 onwards DBH was used for size measurements. All three clones generally grew well across the growing seasons (**Figure 4**). Analysis of tree size by clone and construct showed that in 2018 most events in each clone were performing well (**Supplementary Figure 1**). By the 2016 growing season most areas of the plantation were showing canopy closure, meaning that the branches of neighboring trees overlapped. Very soon after planting it became obvious that tree performance varied widely by location (**Figure 3**). Even in 2017 some low productivity areas still have bare ground visible, such as in the most northern block of male clone 353, indicating that even weeds do not grow well in these locations. Other regions had very large trees and extensive growth of all vegetation, making weed control a constant management challenge.

As the trees became larger differences in performance between neighboring construct pairs became increasingly obvious, indicative of construct and event differences. For example, it was noticed early on that some events from the RNAi-*FT* construct were very small (**Figure 5**), despite being located in areas of the plantation where neighboring trees grew well. In addition to their shorter height, these trees also had short internodes, giving them a bushy appearance. Similar results were observed for all three poplar. The RNAi-*FT* had been designed with the hope of obtaining delayed floral onset, without reductions in vegetative performance. When the work was initiated, the endogenous function of *Populus FT* homologs was unknown. While overexpression of either *PtFT1* or *PTFT2* could lead to early-onset of flowering (Bohlenius et al., 2006; Hsu et al., 2006) the genes have divergent functions, with *PtFT1* controlling the onset of flowering, and *PtFT2* controlling vegetative growth (Hsu et al., 2011). Given that *PtFT1* and *PtFT2* are 89.1% identical at the transcript level, it is very likely that both are being suppressed by the RNAi construct.

A main goal of this study was to identify gene targets and methods (RNAi, DNM, overexpression) that would be useful for genetic containment by leading to prevention or long term delay in the onset of flowering. Trees were screened yearly for the presence of floral buds (before leaf flush), and dormant floral buds were first observed in January 2014 (**Supplementary Figure 2**). Each tree in the plantation was visually screened, and if at least one floral bud was observed then the tree was designed as flowering. If no floral buds were observed then the tree was designated as non-flowering. Colored flagging was used to mark flowering trees in the field; when floral buds flushed trees were re-evaluated for flowering as open flowers are larger and easier to identify than closed floral buds. Yearly floral scoring showed that both female clones started flowering in 2014, while male clone 353 started flowering in 2015 (**Figure 6**, **Supplementary Table 2**). Female clone 6K10 underwent noticeable increases in flowering each year, with 28.8% of trees flowering in 2014, which peaked at 86.4% flowering in 2017, with a small decrease to 77.8% in 2018. Male clone 353 also increased in flowering per year, with 6.0% flowering in 2015 and 67.6% flowering in 2018. Female clone 717 initiated

TABLE 2 | Construct names and genes targeted.

Construct name	Field ID	Construct type	Predicted outcome	Gene(s) targeted or utilized
Control	CTR	Control	No change	None
AGL24-OE	A0	OvExp	Early flowering	AGL24
AG-M2	AM2	DNM	Delayed flowering	AtAG
AG-M3	AM3	DNM	Delayed flowering	AtAG
AP1-M2	AP2	DNM	Delayed flowering	AtAP1
AP1-M3	AP3	DNM	Delayed flowering	AtAP1
FT	FT	RNAi	Delayed flowering	FT1, FT2
SVP-OE	PS	OvExp	Delayed flowering	SVP
AGL20	A20	RNAi	Delayed flowering	AGL20 (SOC1)
AGL24	A24	RNAi	Delayed flowering	AGL24
FT:AGL20:FPF1	FAP	RNAi	Delayed flowering	FT1, FT2, AGL20 (SOC1), FPFL1
FT:AGL20	FA20	RNAi	Delayed flowering	FT1, FT2, AGL20 (SOC1)
PFPFL1	FPI	RNAi	Delayed flowering	PFPFL1
PFPFL2	FP2	RNAi	Delayed flowering	PFPFL2
PTAG	PTG	RNAi	Sterile flowers	AG1, AG2
MpTAG	MPG	RNAi	Sterile flowers	AG1, AG2 (mar)
PTAP:PTAG	PAG	RNAi	Sterile flowers	AP1-1, AP1-2, AG1, AG2
PTAP:PTLF	PAF	RNAi	Sterile flowers	AP1-1, AP1-2, LFY
PTAP	PAP	RNAi	Sterile flowers	AP1-1, AP1-2
PTD	PTD	RNAi	Sterile flowers	AP3
PTLF:PTAG	PFG	RNAi	Sterile flowers	LFY, AG1, AG2
Triple	TRP	RNAi	Sterile flowers	LFY, AG1, AG2, AP1-1, AP1-2
PTLF	PLF	RNAi	Sterile flowers	LFY
PTLF+PTAG	PFPG	RNAi	Sterile flowers	LFY, AG1, AG2

Each genetic construct was given a unique construct name, which appeared as a shortened version on tags in the field (field ID). There were three types of constructs, OvExp, over expression; DNM, dominant negative mutation; RNAi, RNA interference. Non-transgenic control trees were indicated by control (CTR), meaning they did not undergo transformation with a genetic construct. DNM constructs were based on modified versions of A. thaliana genes predicted to inhibit the activity of their Populus homologs. The PFG and PFPG constructs were designed to target the LFY and AG genes; for PFG both gene fragments were part of a single hairpin, as were all other multi-gene targeting constructs. For PFPG two hairpins were present.

flowering in 2014 with 1.0% of trees flowering, then showed 40.5% flowering in 2015, and by 2018 82.0% of trees flowered. The percentage of events flowering per year (events with at least one flowering tree were designated flowering) was generally similar to the percentage of trees flowering per year, with 87.2, 91.5, and 98.6 of events in clones 353, 717, and 6K10 flowering in 2018, respectively (**Figure 6**).

Tree flowering was impacted by tree location, which greatly affected rate of growth across the plantation. Mapping of tree size and tree flowering by location indicated a trend for larger trees tending to flower earlier and heavier (**Figure 7**), though there were also exceptions. A diagonal stripe of higher fertility soil runs southwest to northeast across the plantation, and thus had most of the larger and more intensely flowering trees. There were also regions of the plantation, however, such as the southwest corner, that showed good tree growth but little observed flowering, despite having a mix of constructs and events in the area. Other locations had smaller trees, such as the southeast region, but copious floral production. Our results showed that soil quality likely had complex effects on the onset of flowering beyond that due to growth rate alone.

Starting in 2016 relative floral abundance was scored for each tree, ranging from no flowers (score of 0), to copious flowers across the entire canopy (score of 5); the full scoring system is given in **Figure 7**. Analysis of floral abundance by construct allowed for the identification of constructs and events with reduced flowering. For example, constructs overexpressing *SHORT VEGETATIVE PHASE* (*SVP*) or a dominant negative version of the *A. thaliana APETALA1* gene (*AP1*), or RNAi-suppressing the *AGL24* gene, had events with large trees that flowered very little or not at all, even when neighboring trees flowered heavily (**Figure 8**). Analysis of the relative floral abundance across SVP-OvExp events in clone 6K10, our poplar clone with the highest percentage of flowering events (**Figure 6**), showed that most of these events had little flowering, even in 2018 when essentially all events from controls and normal flowering-onset constructs had flowered (**Figure 9**). By contrast, events from the TRP construct, which was designed to disrupt floral structure not onset, had very abundant flowering per event.

A second main goal for this study was to identify constructs that led to altered (ideally sterile) inflorescences or floral organs. As the flowers tended to open more or less simultaneously per clone and were only open for a brief amount of time in the field during the rainy and cold Oregon winter, branches with dormant floral buds were collected during winter and

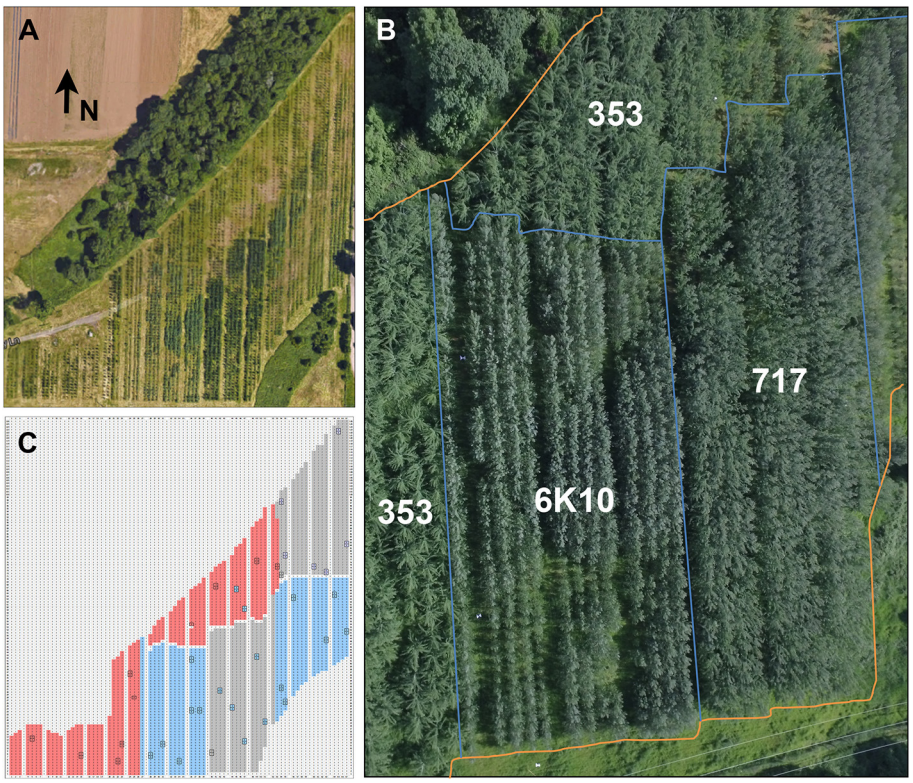


FIGURE 3 | Plantation overview. **(A)** The plantation consisted of 3.6 hectares of hybrid poplar trees surrounded by greenbelt and agricultural areas. The entire plantation was enclosed in deer exclusion fencing. Arrow indicates the direction North (N). Note the variation in foliage color. **(B)** An overhead view of most of the plantation showing the locations of the blocks of each clone. Blue lines show clone boundaries, orange lines show plantation perimeter. **(C)** A graphical representation of the plantation showing the location of each clone. Locations without trees (wide rows, unplanted areas) are shown in white, male clone 353 in red, female clone 717 in gray, and female clone 6K10 in blue. Locations of non-transgenic control trees are boxed in black. Each clone was planted in two blocks, with trees from male clone 353 concentrated on the windward side of the plantation to serve as a pollen source for female clones 717 and 6K10.

flushed in a warm laboratory for initial screening of floral form (**Supplementary Figure 2**). Floral buds are larger than vegetative buds and can be easily recognized in the field. The large majority of flowers observed in the lab were similar to those of control trees. However, some events from RNAi constructs targeting the *LEAFY* (*LFY*) and *AGAMOUS* (*AG*) genes had noticeably different floral forms. Some RNAi-*LFY* events had female flowers with no externally visible carpels and were determined to be sterile (Klocko et al., 2016). Select RNAi-*AG* events had female catkins which opened early and appeared to be larger than control catkins, some of which were also determined to be sterile. Data from the lab were then used to identify constructs and events of interest for observation in the field.

Observation of floral form in the field showed that events had similar phenotypes in the field as they did in the lab. In addition, events with strong floral modifications had stable phenotypes across growing seasons (**Figure 10**). Other events from the RNAi-*AG* constructs had intermediate floral phenotypes (**Figure 11**), and flowers from these events continued to show floral variability, such as mixtures of fertile and sterile capsules on single catkins (green vs. yellow capsules, **Figures 11E,F**). Variation was also observed between male and female clones transformed with the same construct. The same RNAi-*LFY* construct which led to strong female sterile phenotypes gave rise to bisexual or female flowers in male clone 353 (**Figure 12**). Two other constructs targeting *LFY,* either singly or together with the *AG* or *AP1* genes, also led to floral alterations in this clone. Overall, 11 constructs of the 23 tested led to alterations in floral morphology or floral timing in at least one clone (**Table 3**).

Part of the permit requirements for allowing flowering at the field site was a yearly analysis of seed production, seed viability, and frequent screening for the establishment of seedlings in and around the field location (leaf morphology is distinct from wild poplars for the tested clones). Each year catkins from all flowering female clones and constructs were sampled and screened for the presence of seeds, and seeds tested for viability in lab conditions (**Supplementary Tables 3**, **4**). From 2014 through 2017 a total of 300 seeds from female clone 6K10 were found, and a total of 140 seeds from female clone 717 were found. All seeds found were tested for viability by germination testing. For female clone 6K10 the percent germination ranged from 0% of the 10 seeds found in 2014 to 21.7% of the 106 seeds found in 2016, with an overall germination rate of 13.7% for all seeds found in all years. For female clone 717 the percent germination ranged from 5.6% of

TABLE 3 | Construct names and observed outcomes.

Construct name	Predicted outcome	Observed outcomes by clone		
		353	717	6K10
Control	No change	Normal	Normal	Normal
AGL24-OE	Early flowering	Normal	Normal	Normal
AG-M2	Delayed flowering	Normal	Normal	Na
AG-M3	Delayed flowering	Normal	Normal	Normal
AP1-M2	Delayed flowering	**Delayed**	**Delayed**	Na
AP1-M3	Delayed flowering	**Delayed**	**Delayed**	**Delayed**
FT	Delayed flowering	Normal	Normal	Normal
SVP-OE	Delayed flowering	**Delayed**	**Delayed**	**Delayed**
AGL20	Delayed flowering	Normal	Normal	Normal
AGL24	Delayed flowering	Normal	Normal	Normal
FT:AGL20:FPF1	Delayed flowering	Normal	Normal	Na
FT:AGL20	Delayed flowering	Normal	Normal	Na
PFPFL1	Delayed flowering	Normal	Normal	Na
PFPFL2	Delayed flowering	Normal	Normal	Normal
PTAG	Sterile flowers	**Floral alterations**	Normal	**Female sterile**
MpTAG	Sterile flowers	Na	Na	**Female sterile**
PTAP:PTAG	Sterile flowers	Normal	Normal	**Floral alterations**
PTAP:PTLF	Sterile flowers	**Bisexual**	Normal	**Floral alterations**
PTAP	Sterile flowers	Normal	Normal	Normal
PTD	Sterile flowers	Normal	Normal	Normal
PTLF:PTAG	Sterile flowers	Normal	Normal	**Floral alterations**
Triple	Sterile flowers	Normal	Normal	Normal
PTLF	Sterile flowers	**Male sterile, bisexual, female**	Normal	**Female sterile**
PTLF+PTAG	Sterile flowers	**Male sterile, bisexual**	Normal	**Floral alterations**

Flowers and annual onset of flowering from all constructs and clones were scored. Normal, normal form and onset; delayed, late onset; floral alterations, organ identity changes without loss of fertility; bisexual, male and female organs on male clone; female, female organs on male clone; sterile, loss of ovules or pollen; NA, not applicable as no events were planted for that construct and clone. Bold letters indicate modified floral phenotypes.

the 18 seeds found in 2017 to 50.0% of the 2 seeds found in 2014, with an overall germination rate of 28.6% for all seeds found in all years. In addition to laboratory seed testing, the field site itself and the surrounding perimeter were checked for seedlings. No transgenic tree-derived seedlings were identified in the field site or the surrounding perimeter.

Poplar trees can also spread by means of vegetative propagation. Therefore, the site and surrounding perimeter were regularly monitored for the presence of vegetative sprouts, termed suckers. All planted trees had a shade cloth and metal field tag and were planted in a gridded spacing, allowing for the identification of any unplanted poplar shoots. Such vegetative suckers were rare, and were killed when found by spraying them with herbicide, uprooting the stem, and burning the plant material. Low numbers of suckers were found in the field site itself, and all were devitalized shortly after discovery.

DISCUSSION

The goal of this field trial was to analyze the effectiveness of 23 different genetic constructs and 15 target genes for obtaining delayed or modified flowering in poplar, hopefully enabling a high level of genetic containment. Ideally, such trees would have either delayed floral onset or reduced floral fertility without negative impacts on vegetative performance. It was clear that tree growth was uneven across the field site (**Figures 3**, **7**). The site used for the field plantings was previously used for residential and agricultural purposes, and there may be foundation remains, gravel, soils of varying past fertilization, and compacted soil or buried debris, any of which could impact tree performance. The site was also characterized by strips of variable natural soils as a result of past floods and variable sedimentation by the nearby Willamette River. This variation in growth complicated the interpretation of vegetative performance. However, when averaged over the dozens to thousands of trees studied it was clear that the large majority of trees grew well without regard to construct (**Supplementary Figure 1**), and by 2016 most were of a substantial size (**Figure 4**).

Yearly scoring of the flowering which started in 2014 provided us with five years of floral onset data for analysis (**Figure 6**). All trees were planted at the same time and were the same age. The three clones varied in the timing and abundance of floral onset, with female clone 6K10 showing the earliest and highest initial percent of flowering, and male clone 353 showing the latest flowering (**Figure 6**). For all clones, the percent of flowering tended to increase with tree age, as would be expected. Ideally,

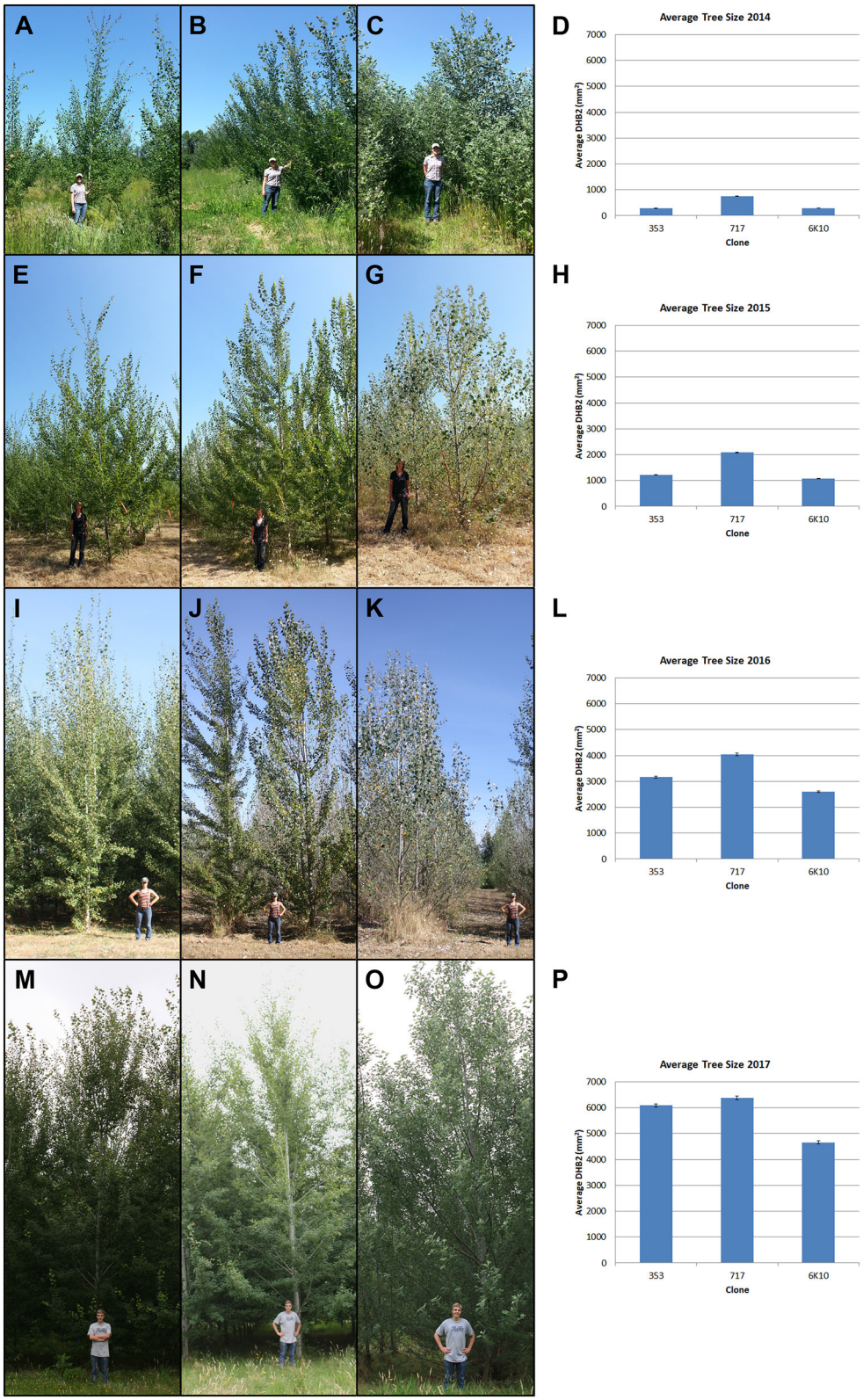


FIGURE 4 | Stages of tree growth for the three tested clones. **(A–C)** Clones 353, 717 and 6K10 with field manager Kori Ault in June 2014. **(E–G)** Clones 353, 717 and 6K10 with field student Anna Magnuson in August 2015. **(I–K)** Clones 353, 717 and 6K10 with field student Lauren Yap in August 2016. **(M–O)** Clones 353, 717 and 6K10 with field student Thomas Howe in June 2017. Graphs show average tree size by clone, as determined by DBH^2, in **(D)** 2014, **(H)** 2015, **(L)** 2016 and **(P)** 2017. Bars show standard error of the mean of all trees per clone.

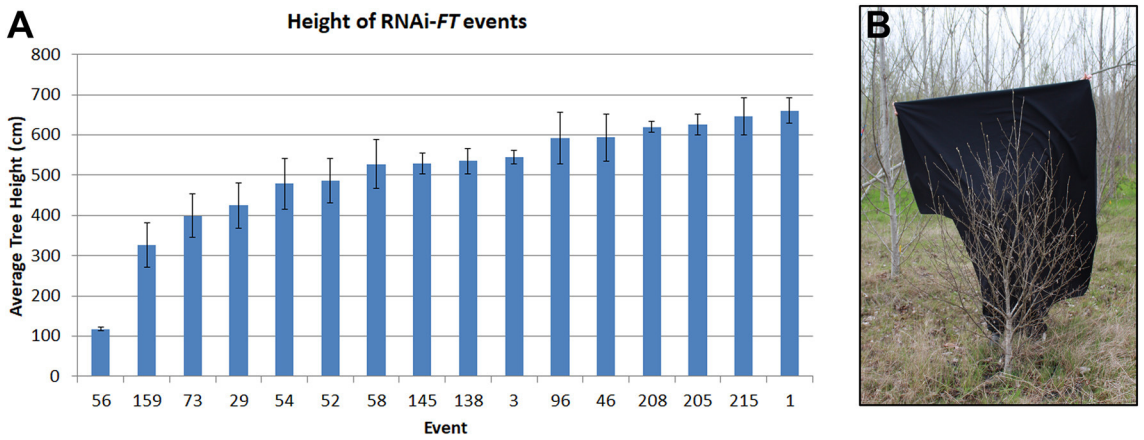


FIGURE 5 | RNAi of *FT* genes led to some dwarf trees. **(A)** Average height of RNAi-*FT* events in female clone 717 as measured in 2015. Bars show average tree height for each event; standard error of the mean is shown. **(B)** Some RNAi-*FT* events showed greatly reduced vegetative growth, with a shorter height and copious branching, as compared to neighboring trees; event 56 from clone 717 is shown. Image from February 2017.

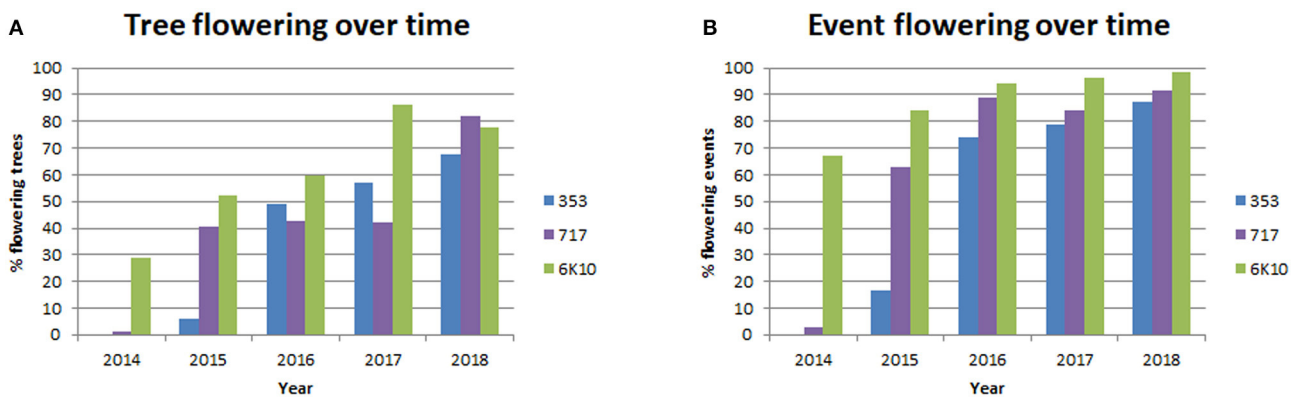


FIGURE 6 | Tree flowering increased with time. Trees initiated flowering in 2014 and each tree was scored yearly for the presence or absence of floral buds. Events were considered flowering if at least one tree from the event had floral buds. Bars show the percentage of tree flowering **(A)** and the percentage of event flowering **(B)** for male clone 353 (blue bars), female clone 717 (purple bars) and female clone 6K10 (green bars).

all trees from a given clone would have relatively synchronized flowering, allowing for easy identification in alterations of floral timing. However, we found that tree location greatly impacted tree performance. For example, while female clone 6K10 flowered the most abundantly of all three clones (**Figure 6**), portions of one block had very low numbers of flowering trees, likely due to variability in the soil quality at that position (**Figure 7**).

We also noticed that the amount of flowers present on each tree varied greatly. Starting in 2016 we scored the relative abundance of flowers present on each tree. At this time about half of the trees, across all three clones, were flowering (**Figure 6**). The variation in tree flowering across the site (**Figure 7**) added to the complexity of determining which constructs and events were leading to delayed flowering or decreased floral abundance. Therefore, we focused on identifying constructs and events with low rates of flowering, or low floral abundance, particularly if trees from such events were located next to other trees with abundant flowering. We found that three constructs most clearly led to delays in floral onset or a decrease in overall floral abundance (**Table 3**). Importantly, based on visual inspection these trees had normal productivity. We found that overexpression of *SVP*, or DNM versions of *A. thaliana AP1* or RNAi of the *AGL24* gene, led to trees that had reduced floral abundance or flowered years later than neighboring trees (**Figures 8**, **9**).

Many of the constructs studied were designed to allow flowering, but to alter floral structure to impair formation of pollen or seeds (**Table 2**). We found that targeting of the *LFY* or *AG* genes led to altered, potentially sterile flowers in female clone 6K10 (**Figure 10**, Klocko et al., 2016; Lu et al., 2018). When the floral alterations were strong and the floral phenotype uniform, these traits were stable across flowering seasons (**Figure 10**), while intermediate traits continued to show variability (**Figure 11**). Events with strong and stable traits would

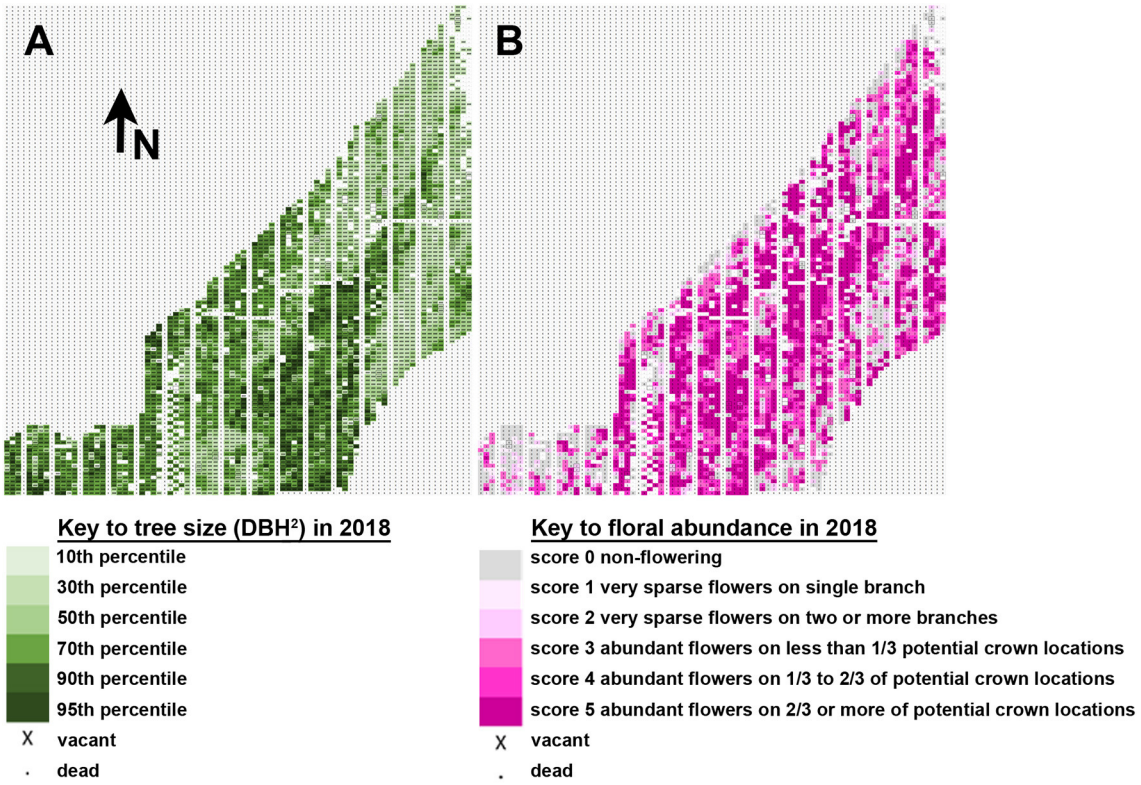


FIGURE 7 | Tree location and size influenced flowering. **(A)** Map of tree flowering observed in 2018. Each rectangle indicates the position of one tree. Tree size is shown on a relative scale from smallest trees in the 10th percentile (pale green) to largest trees in the 95th percentile (dark green). Arrow indicates the direction North (N). **(B)** Map of floral abundance as observed in spring 2018. Floral abundance is shown on a relative intensity scale from no flowers (gray) to abundant flowers across 2/3 or more of potential crown locations (dark pink).

be the most useful for achieving reliable containment. However, it is estimated that even imperfect sterility would greatly reduce gene flow from GE plantations (DiFazio et al., 2012).

Another key finding from this work was the challenge of predicting outcomes across clones. Ideally, each construct would have comparable impacts in each genetic background. We did find that some constructs, such as SVP-OvExp (**Figure 8**), had similar phenotypic outcomes across clones. However, that was not always the case. The same RNAi-*LFY* construct which led to strong female sterility in female clone 6K10 (**Figure 10**) had variable floral phenotypes in male clone 353 (**Figure 12**). Some RNAi-*LFY* events in this male clone had bisexual flowers, or even female flowers. This sort of floral gender change phenotype was previously observed on female clone 717 trees overexpressing poplar *LFY* (Rottmann et al., 2000).

As part of our regulatory permit, we monitored the spread of the trees locally by vegetative shoots, and by seed formation and seedling establishment. Such data are informative regarding the actual risks of spread by vegetative means or sexual reproduction. We did find a small number of vegetative sprouts very close to plantation trees; these were easily killed by herbicide sprays and uprooting the stems. Regular mowing for weed control was likely a contributing factor to the low observed numbers of suckers, as they would be cut off very low to the ground. Such practices are common in managed tree plantations. Yearly surveys for seeds and seedlings showed that while seeds were formed and some were viable under lab conditions (**Supplementary Tables 3**, **4**), no seedlings were found at the field site. Thus, the possibility of spread into neighboring wild populations by seed dispersal and seedling establishment is very low. This is not surprising as it is well known that poplars require special conditions for establishment due to their very small seeds; this includes moist soils during early stages of growth that are free from competition from fast growing weeds (DiFazio et al., 2012). The continuous grass and weed cover around the plantation, and nearby closed forest or annual agriculture, did not provide such permissive conditions.

For genetic containment systems that are acceptable in commercial forestry, it is essential that the genes employed do not adversely affect vegetative growth. Although most of the tested constructs had no detectable effects on vegetative growth, we found that some RNAi-*FT* events were dwarfed in size and had altered vegetative form (**Figure 5**). At the time the work was initiated, it was not known that the two poplar *FT* genes had divergent functions, or indeed that there were two *FT* genes. The small size and altered form of some RNAi-*FT* trees indicate that the *FT2* was likely suppressed, and this gene is important for vegetative performance (Hsu et al., 2011).

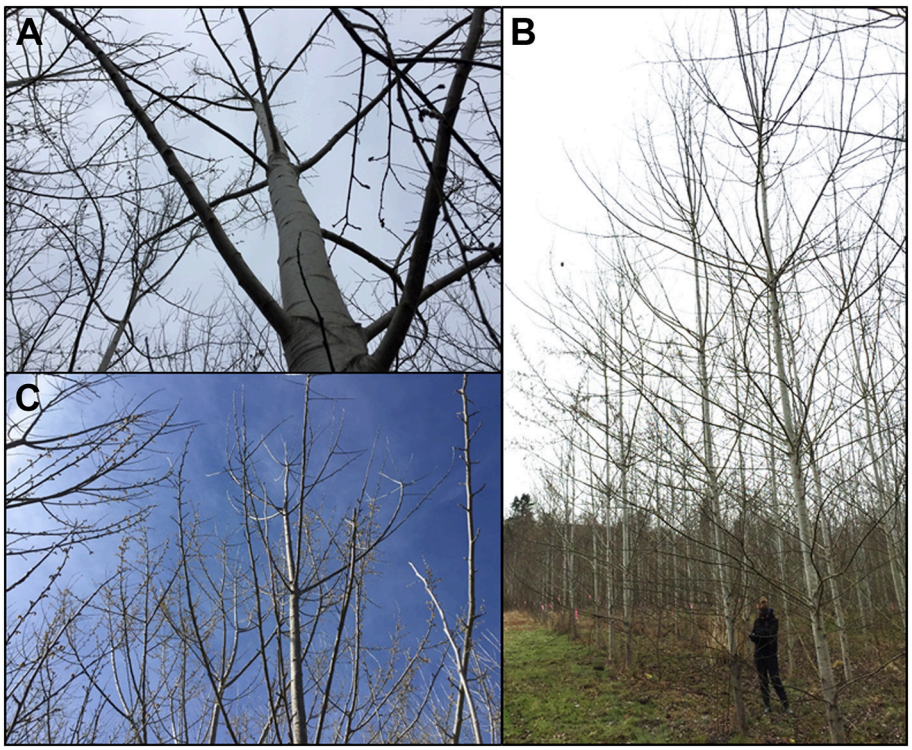


FIGURE 8 | Overexpression of floral suppressor genes prevented or postponed the onset of flowering in poplar. An eight-year-old field trial (photos taken in March 2018) showed many examples of large trees from constructs and events with little or no flowering when nearby trees flowered heavily. **(A)** View up the trunk of a large non-flowering tree from RNAi of poplar AGL24 in male clone 353. **(B)** Overview of plantation row of female clone 6K10 with a pair of non-flowering ramets (foreground) expressing a DMN version based on the *A. thaliana AP1* gene (construct AP1-M3); neighboring flowering trees are visible further down the row. **(C)** A large non-flowering tree (center) from an SVP-OvExp event in female clone 6K10, surrounded by flowering trees.

One challenge for this trial was managing the large number of trees that needed to be monitored over several years of study. This is a result of the variability in RNAi suppression or overexpression among gene insertion events (requiring as many events as possible to see a range of effects), the desire to study male and female flowers, the inclusion of normal and early flowering poplar clones, environmental variation in the plantation as discussed above, and the multiple year delay until onset of flowering in these trees. In total we tested 948 independent transformation events over 8 growing seasons (**Supplementary Table 1**). As we also sought to obtain replicate trees from each event, the numbers of trees needed for analysis was multiplied about four, for a total of 3,315 trees. The variability of RNAi effectiveness among events also means that some constructs could have led to sterile or delayed flowering had additional events been analyzed. For example, no events with altered flowers were observed for trees transformed with the TRP construct, which was designed to suppress the *LFY AG* and *AP1* genes simultaneously from a single hairpin (**Table 2**). However, obtaining strong suppression for all five targets (both *AG* and *AP1* are duplicated in poplar genome) might have required that we test many dozens or even hundreds of events; this was beyond our capability and resources. For goals such as multiple gene knockouts, gene editing technology, especially CRISPR, should be far more efficient, and knock-outs can be identified in the laboratory and only a small sample propagated and planted in the field. They are also likely to be far more stable than gene suppression or overexpression technologies, enabling confident genetic containment and thus improving public acceptability and simplifying regulatory decisions.

Our data show that suppression of the *LFY* and *AG* genes with other RNAi constructs led to floral alterations (**Figures 10–12**, **Table 3**), but for some reason combinatorial constructs were unsuccessful in this study. It is likely that the type of RNAi construct affects the rate of multiple gene suppression. For example, we tested two different constructs to simultaneously suppress the *LFY* and *AG* genes (**Table 2**). The PFPG construct had two hairpins, one for *LFY* and one for the *AG* genes, and the PFG construct had a single hairpin containing both inverted repeats. The two hairpin construct led to floral alterations and the single hairpin construct resulted in normal flowers in male clone 353 (**Figure 12**). The two hairpin PFPG construct also led to floral alterations in female clone 6K10, but the single hairpin also did (**Table 2**).

A second challenge from this trial was related to the sheer size of the site, the number of trees, and multiple-year duration of the trial. In addition to the expected challenges of weed control and irrigation, damage to trees from biotic sources was a persistent

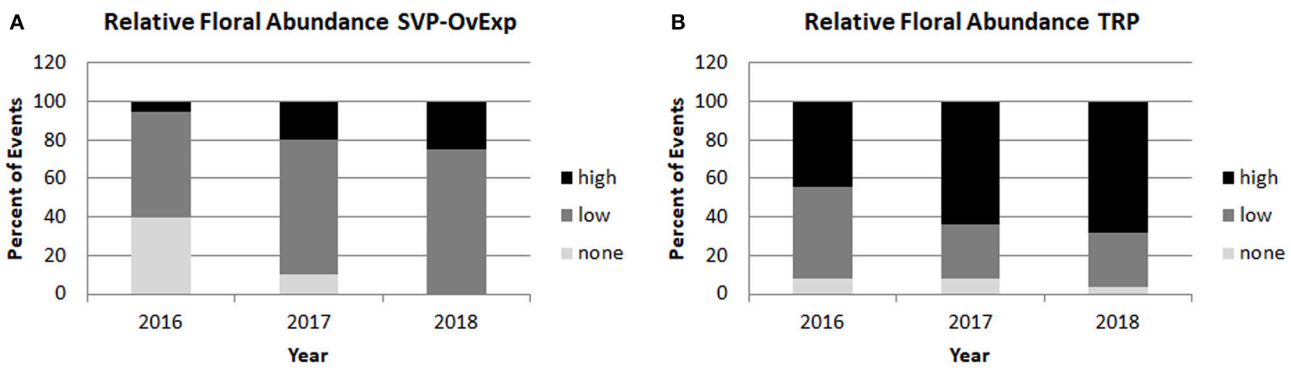


FIGURE 9 | Overexpression of floral suppressor SVP led to reduced floral abundance across events and years. Relative floral abundance was scored yearly for all trees. Percentage of events with average floral scores of 0 (corresponding to no floral buds) were categorized as none, events with average floral scores of less than 3 (meaning less than 1/3 of the crown had copious floral buds) were categorized as low, and events with average floral scores of 3 or higher (meaning at least 1/3 to the entire crown had copious floral buds) were categorized as high. Yearly floral abundance data from clone 6K10 events transformed with **(A)** and SVP-OvExp construct or **(B)** an RNAi construct targeting the *LFY, AG.* and *AP1* genes that did not affect floral onset are shown.

challenge. Deer were found to be particularly tricky adversaries, capable of squeezing under fence lines. With over 3.5 hectares of trees to hide in and no predators, our trial also provided the deer with an excellent source of shelter and food. We also found that shade cloths placed under each tree for weed suppression were utilized by rodents for cover, and often damaged trees by girdling (**Figure 1**). Human vandals were a more worrisome but thankfully less frequent source of damage; the most recent harm to our trees occurred in 2001 (**Figure 1**), and no damage has occurred since.

The large size and delayed flowering of clones 353 and 717 made floral collections challenging. Dormant floral bud sampling in 2016 and 2017 required a pole pruner that included a set of clippers located at the end of an extendable pole. Tree size will also present a continuing challenge at the time of trial termination. Once a field trial is complete, all trees must be killed and the area monitored until no new sprouts have been observed for two full years. This task can be quite daunting for poplar trees, which are extremely good at re-sprouting from their roots, even after herbicide treatment of stumps or sprouts. Carefully chosen herbicides, applied at the optimal times of year, and some years of retreatment of sprouts, are likely to be needed based on our past experience.

Obtaining and maintain regulatory approvals for a flowering field trial of trees is difficult; most researchers do not attempt it. However, as modification of fertility was the point of the study, there were far too many large trees to consider bagging of all flowers, and performance of containment technology under natural plantation conditions was our goal, there was no choice but to seek approval for normal flowering. Fortunately, the use of aspen/white poplar clones that are not compatible with native cottonwood *Populus trichocarpa*, the very specialized establishment needs for poplar, and the innate biosafety of tree sterility traits (and potential containment benefits in the future) prompted USDA to agree that our field trial was safe to conduct. The need for *any* containment for a field trial of containment genes seems absurd to us, but is the product of a system that is focused on the method of modification and the vectors and genes used, not the novelty and risks nor the potential benefits, of the resulting traits. However, obstacles to field trials of GE

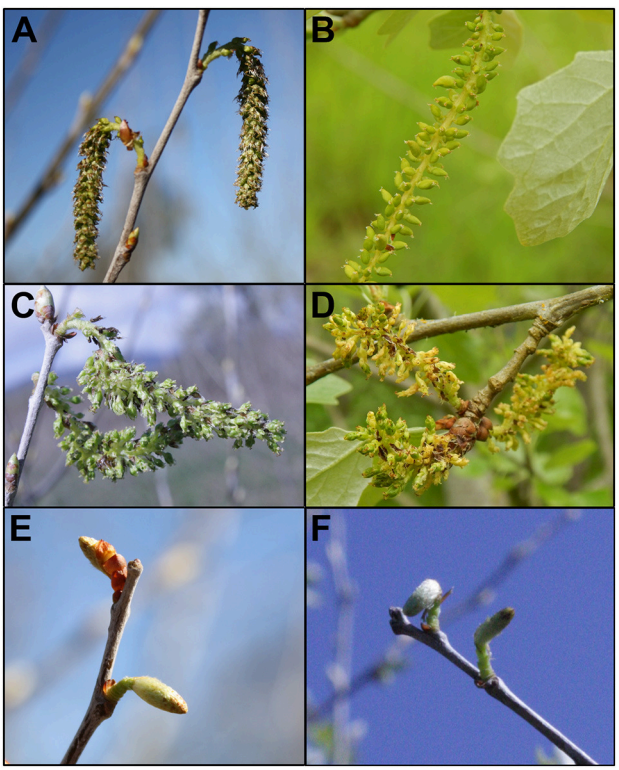


FIGURE 10 | Events with strong floral phenotypes were stable across flowering seasons. Flowers from wild type female 6K10 showed similar catkin formation in **(A)** 2014 and **(B)** 2017. Flowers from RNAi-AG (mar) event 165 showed catkins with replicated carpels in **(C)** 2014 and **(D)** 2017. Flowers from RNAi-LFY event 139 showed small catkins with no externally-visible carpels in **(E)** 2014 and **(F)** 2015.

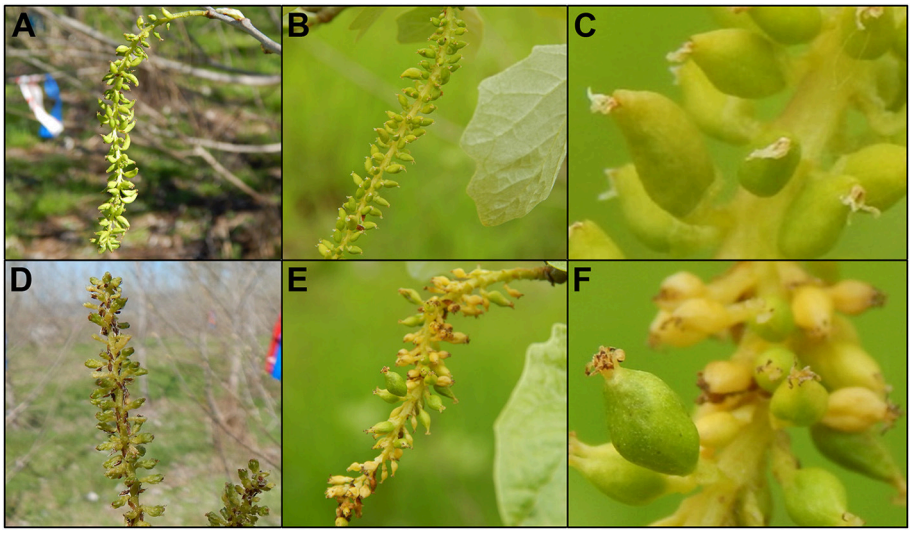


FIGURE 11 | Trees with intermediate levels of RNAi suppression had catkins with variable phenotypes. Flowers from 6K10 control trees in **(A)** 2016, **(B)** 2017 showed well-formed carpels. **(C)** Carpels were of uniform appearance across the catkin. Upright catkins from RNAi-*AG* (mar) event 119 from **(D)** 2016 and **(E)** dangling catkins from the same event in 2017. **(F)** Carpels from these flowers had non-uniform sizes, colors or shapes.

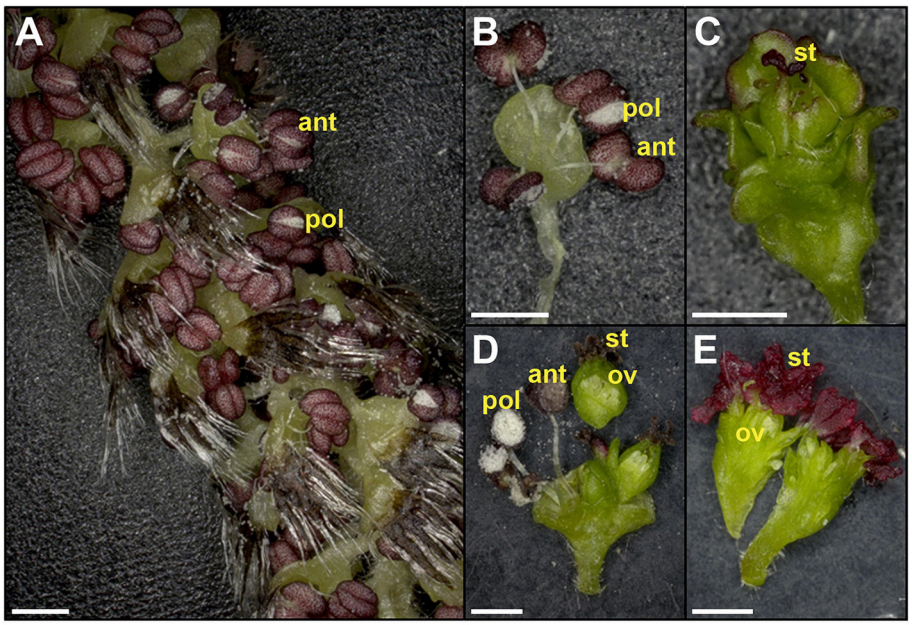
trees are much more severe in many other parts of the world (Viswanath et al., 2012); we are fortunate to have a workable, science informed system in the USA. Nonetheless, we devoted substantial effort to producing numerous permit applications, reports, and undergoing inspections that are very difficult for most academic and public sector laboratories to afford.

In sum, we obtained valuable lessons about gene function, stability of trait expression, and containment options from our multiple-year field trial. All of these lessons support the finding that GE methods of genetic containment, specifically RNAi and overexpression, can be very effective and reliable for reducing risks of gene flow. Our results have identified several genes and types of genetic modifications that warrant further study given our findings. Future work will hopefully include a larger number of years that more closely approximate the commercial lifetime of plantation tree varieties, and examination of larger numbers of insertion events, especially for the RNAi constructs. The *AG* and *LFY* genes, in particular, appear to be very promising targets for bisexual sterility without obvious impacts on vegetative development; however, their impacts and performance in male clones is unclear, perhaps due to a lower rate of RNAi suppression in the male clone 353-53. The targeting of both of these genes with CRISPR is expected to be feasible and highly successful, establishing whether gene knock-down would indeed be a universal containment technology in poplar. Likewise, promoter editing of the *SVP* and other floral-onset suppressive genes might be superior to generic overexpression, and highly successful means for maintaining trees in a juvenile state to promote rapid growth and avoid flowering. The growing genomic and molecular knowledge of trees, combined with the precision of gene editing, suggest that many new and more powerful genetic innovations are just around the corner.

MATERIALS AND METHODS

Construct Assembly

RNAi constructs were produced based on *Populus* sequences available at the time, which included partial to full-length cDNAs and the initial *P. trichocarpa* genome release. Gene fragments (**Supplementary File 1**) were cloned in the sense and antisense directions into the pHannibal vector (Wesley et al., 2001) creating a hairpin, prior to subcloning into the binary vector pART27. Hairpin expression was controlled by the *Cauliflower mosaic virus* 35S promoter, and the *Agrobacterium* tumefaciens octopine synthase (OCS) terminator. For RNAi constructs targeting unrelated genes, fragments of the targeted genes were first assembled in pBluescriptKS and the chimeric fragment then used to generate an RNAi transgene as described above. For the mPTAG vector, the RNAi transgene was inserted into the Not1 site of pG3KM (Li et al., 2008) and then the region between the TDNA borders excised with Acs1 and inserted into a modified pART27 vector (pART27A) where the TDNA region between the Not1 sites had been removed and replaced with an Acs1 linker. Dominant negative (DMN) constructs were alterations of the MADS-domain sequence based on previously described changes (Jeon et al., 2000). The M2 mutation of *AGAMOUS (AG)* and *APETALA1 (AP1)* was alteration of amino acids 30 and 31 from KK to EE, the M3 mutation of *AP1* and *AG* was alteration of amino acids 24 and 25 from RR to LE. The DNM transgenes were controlled by the double enhancer 35S promoter and the *Pisum satvia* E9 terminator. The DNM expression cassettes were assembled in pG3K (Li et al., 2008) and then the DNM and selectable marker transgenes were excised as a single fragment by Acs1 digestion and inserted into pART27A. Overexpression constructs were assembled in pCAPO, which is identical to the previously described pCAPT (Filichkin et al., 2006) except that



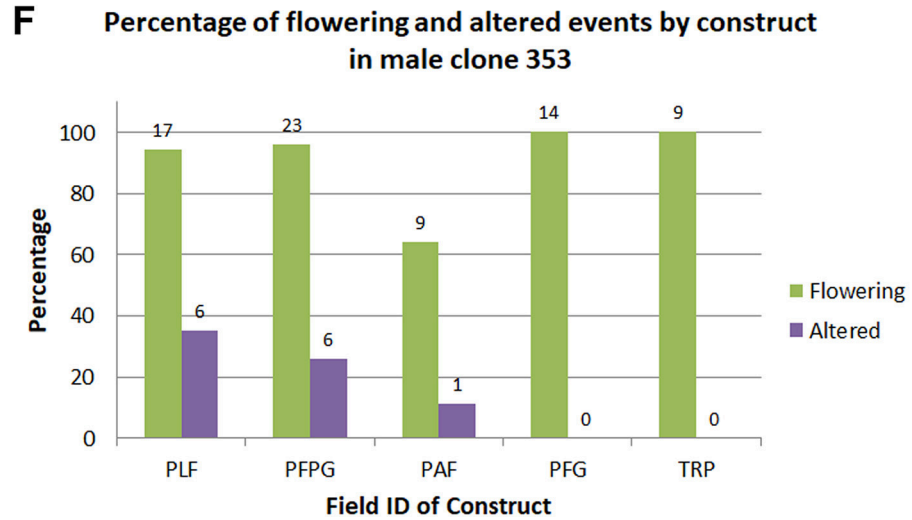


FIGURE 12 | Male clone 353 had variable floral phenotypes from constructs giving strong female sterility. Normal looking **(A)** catkin and **(B)** flower from RNAi-LFY/AG event. **(C)** Sterile male flower from RNAi-LFY/AG event. **(D)** Dissected bisexual flowers with ovules from RNAi-LFY event. The carpel in D was sectioned and placed above the rest of the flower to better display the ovules. **(E)** Dissected female flowers with ovules from RNAi-LFY event. ant, anther; pol, pollen grains; st, stigma; ov, ovule. Scale bars denote 1 mm. **(F)** Graph of flowering events and floral morphology in male clone 353 from 2018. Bars show the percentage of all flowering events (green bars) and events with floral alterations (purple bars) from RNAi-*LFY* and related constructs. PLF, RNAi-*LFY*; PFPG, RNAi-*LFY*+RNAi-*AG*; PAF, RNAi-*LFY:AP1*; PFG, RNAi-*LFY:AG*; TRP, RNAi-*LFY:AP1:AG*. Numbers above bars denote the number of events.

the antisense fragment of the OCS terminator and PIV2 intron are absent.

Plant Transformation and Field Planting

Constructs were transformed into the three poplar clones using standard transformation methods (Filichkin et al., 2006). Tree propagation and field design were previously described (Klocko et al., 2016). In brief, rooted trees were planted in 6 blocks, such that each clone was present in two blocks. Pairs of trees from each transformation event were randomized in that block. Spacing between rows was 2.29 m, with a larger space of 6.10 m after every four rows to allow for vehicle access. Shade cloth was placed under each tree to aid in weed suppression, and each tree was labeled with a metal tag indicating the clone, construct, event and ramet. The field site was drip irrigated the first two summers (2011 and 2012) then discontinued as trees were well established. Weeds were controlled by mowing between rows and using a rotary motorized "weed-wacker" between trees.

Tree Survival and Vegetative Performance

Tree survival was scored each year at the time of vegetative bud flush. Tree size was measured by total height of the stem, and by stem diameter at breast height (DBH), a distance of 137 cm above ground level. Representative stands of each clone were imaged in the summer using a Canon Rebel XSI digital camera as a record of tree size. In spring 2017 an unmanned aerial vehicle (a drone) was used to obtain overhead images of the entire plantation.

Floral Scoring and Indoor Analysis of Floral Form

All trees were scored yearly in January and February for the presence or absence of dormant floral buds. Trees with at least one floral bud were designated as flowering. Trees with at least four branches with one or more buds were sampled by collecting small branch cuttings for floral analysis in the lab. Once flowers flushed in the field trees were rescreened to account for any floral buds missed in the initial survey. Collected twigs were stored at 4 degrees until they were analyzed in batches by clone and construct. Indoor flush was carried out by cutting off the ends of the twigs at a 45° angle and immediately placing the cut ends in cups of water. The plastic cups were inside a plastic bin lined with damp paper towels. Once all twigs were in water the entire bin was tented with a plastic bag to maintain high humidity, cut pieces of bamboo located in each corner of the tub kept the plastic from touching the branches. Branches were incubated at room temperature until most branches had enlarged catkins, about 5 days. Flushed twigs were photographed using a Canon Rebel XSI digital camera. Floral form was initially analyzed in the lab before buds flushed in the field.

Scoring Relative Floral Abundance

Starting in 2016 a floral abundance score was used as a means to categorize relative floral abundance. The entire crown of the tree was surveyed by two researchers, one on the east side of the tree and the other on the west side of the tree. Trees with no flowers were scored 0, trees with very sparse flowers on a single branch were scored 1, trees with very sparse flowers on two or more branches were scored a 2, trees with abundant flowers on less than 1/3 of potential crown locations were scored a 3, trees with abundant flowers on ½ to 2/3 of potential crown locations were scored a 4, trees with abundant flowers on 2/3 or more of the potential crown locations were scored a 5.

Field Analysis of Floral Form Microscopy Keyence Digital Microscope

Flowers that flushed in field conditions were photographed in the field using a Canon Rebel XSI digital camera. Selected flowers were collected, bagged and placed at 4 degrees. These flowers were imaged using a Keyence digital microscope VHX-6000.

Catkin Collection and Seed Presence and Viability Analysis

Starting in 2014, female catkins were collected from female clones 6K10 and 717. Trees were sampled such that catkins from at least two events (if available) were obtained from each construct and clone that flowered in that year. Catkins were collected into small paper envelopes, which were closed in the field then opened in the lab to allow catkins to dry, causing the release of cotton and seeds. Dry catkins were screened for seeds; any potential seeds were removed with tweezers and placed into 1.5 ml tubes until all catkins were screened. Seeds were counted then placed onto damp filter paper in 100 ml petri dishes. Dishes were sealed with parafilm to prevent moisture loss and incubated on the lab bench for 7 days. The number of germinated seeds was counted and tallied. Seeds were scored as germinated by the emergence of a root at least as long as the seed.

AUTHOR CONTRIBUTIONS

AK, AB, and SS wrote the article. AB selected target genes and designed genetic constructs. AK, HL, and SS designed experiments. AK, HL, AM, SS, and CM collected data. AK, HL, AM, and CM analyzed data. Datasets are available on request.

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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.2018.00100/full#supplementary-material

Supplementary Figure 1 | Trees from most constructs performed well in all three clones. Average tree size (DBH^2) of all trees was calculated from measurements collected in early 2018. Graphs show average size of all trees per construct for **(A)** clone 353, **(B)** clone 717 and **(C)** clone 6K10. Note that clone 717 had a single non-transgenic control tree (CTR) which grew poorly. Bars show construct averages across all trees; standard error of the mean is shown.

Supplementary Figure 2 | Dormant floral buds were flushed in the lab for initial floral classification. **(A)** Trees from female clone 6K10 in January 2015, trees with floral buds have blue flagging, trees with buds collected for indoor analysis have

an additional red flag. **(B)** Small twig cuttings with dormant floral (fl) and vegetative (veg) buds. **(C)** Flushed control catkins, **(D)** flushed normal RNAi-LFY catkins, **(E)** RNAi-LFY twigs with very small catkins, **(F)** RNAi-AG (mar) twigs with enlarged catkins.

Supplementary Table 1 | Numbers of trees planted and survival to date by clone and construct. Trees were first planted in 2011 and survival monitored yearly. Current numbers of surviving trees are from the 2017 spring bud flush. Event refers to individual transgenic occurrences; ramets are individual trees, each field ID refers to a unique genetic construct (see **Table 2**).

Supplementary Table 2 | Flowering events by clone, construct and year. Tree flowering was monitored yearly, events with at least one flowering tree were considered flowering. NA for flowering refers to categories where no events were planted for that construct in that clone. Each field ID refers to a unique genetic construct (see **Table 2**).

Supplementary Table 3 | Seed formation and seed viability for female clone 6K10. Yearly surveys checked for seed formation and seed viability from events which flowered.

Supplementary Table 4 | Seed formation and seed viability for female clone 717. Yearly surveys checked for seed formation and seed viability from events which flowered.

Supplementary File 1 | Sequences of gene fragments used to make RNAi constructs. A list of the portions of gene sequences used in creation of RNAi constructs.

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Conflict of Interest Statement: SS has directed a university and industry funded research consortium (TBGRC) based at Oregon State University for more than two decades that contributes to funding of research in his laboratory. Its work is directed at producing solutions to the problems of gene dispersal from genetically engineered and exotic trees.

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

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Bt Eggplant Project in Bangladesh: History, Present Status, and Future Direction

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The purpose of this article is to provide information on the history, accomplishments, and future direction of the Bt brinjal (eggplant) program in Bangladesh, formerly under the Agricultural Biotechnology Support Project II, now the South Asia Eggplant Improvement Partnership (SAEIP). The India-based Maharashtra Hybrid Seed Company (Mahyco) developed an eggplant expressing Cry1Ac (EE-1) for control of the eggplant fruit and shoot borer (EFSB). In a partnership among Mahyco, USAID, Sathguru Management Consultants and Cornell University EE-1 was provided to the Bangladesh Agricultural Research Institute (BARI) who bred it into local varieties. After regulatory approval, four varieties were distributed to 20 farmers who harvested Bt brinjal in 2014. Adoption in subsequent years has increased rapidly so that, in 2018, 27,012 farmers used this technology. This article provides background information on the process leading up to current adoption levels, the level of control of EFSB achieved and the economic benefits of Bt brinjal. Efforts on stewardship, farmer training and communication are discussed. In order to ensure the long-term future of the partnership, we discuss the need to enhance involvement of the private sector in the production and stewardship of Bt eggplant. Bt brinjal is the first genetically engineered crop to be commercially released in Bangladesh, and other GE crops are in the pipeline. Hence, success of the Bt brinjal partnership is likely to affect the future of other GE crops in Bangladesh, as well as other parts of the world where biotechnology is needed for food security and environmental safety.

Keywords: eggplant, brinjal, Bt crops, biotechnology, genetic engineering, fruit and shoot borer, *Leucinodes orbonalis*

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INTRODUCTION

The Problem

Solanum melongena L. (eggplant, also known as brinjal in Bangladesh) is an important, inexpensive, and popular vegetable in Bangladesh, second only to potato in production. It is grown on nearly 50,000 hectares. Its production provides an important source of cash income for small resource-poor Bangladeshi farmers. The biggest constraints to eggplant production are chronic and widespread infestations by the eggplant fruit and shoot borer (EFSB), *Leucinodes orbonalis* Guenée (Lepidoptera: Crambidae). Caterpillars damage eggplant shoots and flowers, but the most serious

damage is caused by their boring into the fruit and rendering it unmarketable. Farmers routinely spray broad-spectrum insecticides, often two to three times per week, and, in some cases, twice a day. Consequently, over 100 sprays per season may be applied, resulting in high residues on the fruit. Farmers lose anywhere from 30 to 60% of the crop yield to EFSB despite the high use of insecticides. The cost of insecticide treatments accounts for 35 to 40% of the total cost of cultivation of brinjal. Such an insecticide-dependent strategy poses both environmental and health concerns.

Creating a Solution

The India-based Maharashtra Hybrid Seed Company (Mahyco) used a *Bacillus thuringiensis cry1Ac* gene to transform brinjal to be resistant to EFSB (Shelton et al., 2017). The *cry1Ac* gene is widely used in Bt cotton and the protein is a component of many organic biopesticides. In all cases, Cry1Ac has a long history of safe use (ILSI CERA, 2010). The resulting GE Bt eggplant (termed "*event*" EE-1) demonstrated control of EFSB and was provided to the Bangladesh Agricultural Research Institute (BARI) through a public private partnership between Mahyco, Cornell University, Sathguru Management Consultants, BARI and the United States Agency for International Development (USAID) under the Agricultural Biotechnology Support Project II cooperative agreement (ABSPII; http://absp2.cornell.edu). BARI subsequently introgressed the EE-1 event into nine local eggplant lines. Breeding and efficacy trials were conducted beginning in 2005 and continue today. The ABSPII project ended in 2014. A new cooperative agreement was awarded in 2015 to scale the improved Bt eggplant to Bangladesh farmers under the South Asia Eggplant Improvement Partnership (SAEIP)(http://bteggplant.cornell.edu).

APPROVAL AND ADOPTION OF BT BRINJAL

Approval Process

BARI applied to the National Technical Committee on Crop Biotechnology (NTCCB) to release Bt eggplant. Following the recommendation from NTCCB, the application for release was forwarded to the NTCCB Core Committee followed by the National Committee on BioSafety (NCB). The Bangladesh government granted approval for release of four varieties (BARI Bt brinjal varieties 1, 2, 3, and 4) for "limited cultivation" in the field on 30 October 2013 (three other varieties are pending and two others are uncertain). On 22 January 2014, Bt seedlings of the four lines were distributed to 20 farmers in four districts.

Rapid Adoption

In 2014–15, BARI provided seeds or transplants to its On-farm Research Division (OFRD) to conduct research/demonstration trials on 108 farmer fields in 19 districts. In 2015–16 and 2016–17, demonstration trials were conducted in 250 farmer fields in 25 districts and 512 farmer fields in 36 districts, respectively. In 2017–18, BARI provided seeds to 569 farmers in 40 districts. In addition to distribution by BARI, seeds have also been distributed to farmers through the Department of Agricultural Extension (DAE) to 6,000 and 7,001 farmers in 2016–17 and 2017–18, respectively, and for sale through the Bangladesh Agricultural Development Corporation to an additional 17,950 farmers in 2018 (**Figure 1**). With an estimated 150,000 brinjal farmers in Bangladesh, the 2018 adoption translates to an estimated ∼17% of brinjal farmers in Bangladesh who are enjoying the benefits of the technology.

PERFORMANCE

Control of EFSB and Effects on Non-target Arthropods

According to BARI reports for 2015 and 2016, the performance of Bt eggplant in demonstration trials was far superior to non-Bt eggplant, with fruit infestations in Bt eggplant ranging from 0.04–0.88% compared to 48–57% in non-Bt eggplant (Mondal et al., 2016).

A separate 2-year experiment (2016–17) conducted by BARI scientists compared the four Bt lines to their isolines, with and without insecticide treatments. Fruit infestation for Bt varieties varied from 0 to 2.27% in 2016, 0% in 2017, and was not significantly affected by the spray regime in either year (unpublished). In contrast, fruit infestation in non-Bt lines reached 36.70% in 2016 and 45.51% in 2017, even with weekly spraying. However, maximum fruit yield was obtained from sprayed plots compared to non-sprayed plots, indicating that other insects including whiteflies, thrips and mites can reduce plant vigor and subsequent fruit weight. This result is not unexpected since, as with other Bt crops, the EE-1 event was not designed to control all insect pests. This trial also assessed potential effects of these Bt brinjal lines on non-target arthropods. Based on other similar studies (Naranjo, 2014; Navasero et al., 2016), it is not surprising that statistically similar densities of non-target arthropods, including beneficial arthropods, were observed in both Bt and non-Bt varieties in most cases.

Economics and Pesticide Use

A study was conducted by BARI scientists in 35 districts during the 2016–17 cropping season using 505 Bt brinjal farmers and 350 non-Bt brinjal farmers (unpublished). Net returns per hectare were $2,151/ha for Bt brinjal as compared to $357/ha for non-Bt brinjal, a 6-fold difference. This study also indicated that farmers saved 61% of the pesticide cost compared to non- Bt brinjal farmers, experienced no losses due to EFSB, and received higher net returns.

Similar economic benefits were obtained in a two-year experiment by another set of BARI scientists who found that a higher return was obtained from the Bt varieties over non-Bt isolines, irrespective of insecticide spray regime (unpublished). Results indicated that high quality EFSB-free brinjal could be produced without insecticide treatments but that insecticide control of "sucking insects" provided even higher economic returns on the Bt lines.

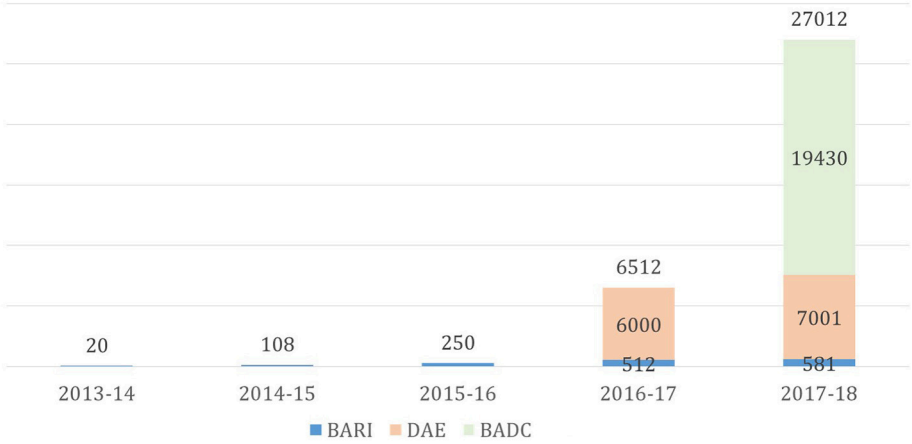

FIGURE 1 | Yearly adoption rate of Bt brinjal in Bangladesh.

STRATEGIES FOR SUSTAINING THE TECHNOLOGY

The economic and environmental benefits of Bt brinjal are clear: enhanced control of a difficult insect pest; reduced use of insecticides and their effects on applicators, consumers, and non-target organisms in the environment; and increased revenue to farmers. Stewardship strategies are needed to sustain these benefits in Bangladesh.

Stewardship

Bt eggplant was the first GE crop released for cultivation in Bangladesh and, accordingly, has provided regulators and farmers with their first experience in managing a GE crop. BARI was designated as the lead Bangladesh institute to produce and distribute Bt brinjal to farmers, and partnered with the SAEIP to ensure stewardship of Bt brinjal in both the pre- and post-launch phases. To ensure long-term durability and success of the technology, the partnership has prioritized efforts to train stakeholders, including researchers, academics, seed production experts, extension professionals and farmers on the need for effective stewardship for Bt brinjal. The team at BARI has been trained on "Excellence Through Stewardship"[1] (ETS), a life cycle approach to GE product management, primarily through the private sector partner, Mahyco, who has extensive experience in managing scaling activities commercially. This includes capacity building efforts to ensure that quality seed—genetic purity, high viability and expression of Cry1Ac—is produced in adequate amounts to meet grower demand.

Other capacity building efforts include development and documentation of standard operating procedures (SOP) for seed testing, proper seed packaging and labeling, and record keeping that meets industry audit standards. The partnership has introduced tools to monitor the production, distribution and inventory management of Bt brinjal seeds as part of the goal to meet international standards. BARI is the sole producer of seed for the four approved Bt brinjal varieties, but has recently provided breeder seed to the Bangladesh Agricultural Development Corporation (BADC) to further increase seed quantities. BARI has distributed seed for free to growers but BADC charges a minimal fee. Of note is that the four Bt brinjal lines that have been released are not hybrids, so growers can save seed, although they are discouraged from doing so for agronomic reasons. Inclusion of the EE1 event in a hybrid background would further increase the yield potential of Bt eggplant.

While stewardship begins with quality seed, other practices are equally vital for the long-term sustainability of Bt brinjal in Bangladesh. Studies have shown that plants derived from EE-1 can be considered as "high expression" plants (Hautea et al., 2016), typically a major component of an effective insect resistance management (IRM) program (Bates et al., 2005). A second common component of IRM is to utilize a refuge of non-Bt plants so that Bt-susceptible alleles can be maintained in the EFSB population. The refuge requirement was set by the partnership at 5% during this initial phase of adoption. A third component of IRM is to develop and utilize baseline studies of susceptibility to Cry1Ac and monitor for any changes that might indicate emerging resistance. Efforts are underway to enhance the existing dataset. A fourth component of IRM is pyramiding Bt genes into plants. While it is recognized that introducing pyramided plants initially would have been desirable, this was not possible when the partnership began, but is being strongly advised for the future.

Farmer Training

Farmer training is the lynchpin of sustainable production of this valuable product in Bangladesh. Prior to the first release of Bt brinjal in 2014, farmer training was conducted by BARI, and BARI continues to be the institution responsible for training. Bangladeshi farmers are well versed in growing brinjal, so

[1]http://www.excellencethroughstewardship.org/

training is focused 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."

BARI continues training efforts through hundreds of OFRD farm trails mentioned above in dozens of districts in Bangladesh where brinjal is grown. In addition to BARI, the Department of Agricultural Extension (DAE) and the Agriculture Information Service (AIS) have more recently become involved in training and distributing information on Bt brinjal. These units have their own facilities and personnel for farmer training. The partnership is in a position to help support their efforts to meet this increased demand for information.

Communication Efforts

Not surprisingly, the introduction of Bt brinjal has a strong following in the domestic and international media. Farmer use and satisfaction with Bt brinjal is reflected in the increasing number of positive press releases. Proactively the partnership has worked to provide access to press releases highlighting factual information for stakeholders such as the 2016 studies conducted in the Philippines that showed nearly 100% control of EFSB by Bt brinjal (Hautea et al., 2016) and no negative impacts on non-target arthropods (Navasero et al., 2016). Publishing similar results from agronomic studies and socioeconomic studies conducted in Bangladesh (as described above) is a high priority for the project. Such information will also be highlighted in the partnership website (Bteggplant.cornell.edu) which is actively maintained, and through social media. The partnership has developed print and audio-visual materials for information sharing and awareness building. It has also conducted a number of national level workshops to engage stakeholders and policy makers.

The partnership also works closely with the Cornell Alliance for Science (allianceforscience.cornell.edu) which provides factual information about agricultural biotechnology and has been a valuable partner in disseminating information about the partnership. The Alliance has enhanced capacity in social media that benefits the partnership in the short and longer term. Other activities include supporting the global March for Science to increase knowledge about agricultural biotechnology and the role it can play in ensuring food security and environmental protection.

Most importantly, the partnership continues to receive strong support from the Honorable Agriculture Minister Begum Matia Chowdhury, MP. Her words from a workshop held in March 2017 in Bangladesh have made her position clear: "Development of brinjal fruit and shoot insect resistant-Bt brinjal is a success story of local and foreign collaboration. We will be guided by the science-based information, not by the nonscientific whispering of a section of people. Good science will move on its own course keeping the anti-science people down. As human beings, it is our moral obligation that all people in our country should get food and not go to bed on an empty stomach. Biotechnology can play an important role in this effort."

Personnel and USAID

As with any partnership, quality personnel are essential. In the last year our partnership has benefited greatly by a new country coordinator who is well respected as a Bangladesh scientist and within the various Bangladesh agencies involved with the project.

Additionally, a new stewardship coordinator well versed with ETS became part of the team.

USAID is committed to supporting countries that wish to develop and commercialize science-based technologies including GE crops. Bangladesh chose to develop a partnership, first with ABSPII and now SAEIP, to develop and commercialize Bt brinjal to alleviate the overuse of pesticides on this important Bangladeshi crop. The partnership is well positioned to continue to increase adoption and stewardship, as well as evaluate the significant socioeconomic impacts of this technology including the positive human and environmental effects of reduced pesticide treatments. The ultimate goal is that the process and knowledge of this partnership be incorporated into the core practices of the public sector of Bangladesh and the private sector that sells and develops high quality seed.

The Regulatory System in Bangladesh

Bangladesh has a variety-based registration system rather than an event-based system. Thus, the currently approved Bt EE-1 derived lines, and the five others that were developed, must individually be approved after being tested in the field. In contrast, most other countries rely on an event-based approval process. The efficiency and cost of event-based registration helps move a product to market more rapidly. Numerous studies have shown this process does not compromise efficacy or safety. Discussions are underway in Bangladesh that may help them adopt an event-based system to allow more rapid development of lines developed by BARI and the private sector.

There is also a need to assess compliance of regulations affecting Bt brinjal at all levels from the laboratory to the field, and this could be a function of ETS. Likewise, efforts should also explore the potential use of "refuge in the bag" (i.e., a specific mix of Bt and non-Bt seed in the same container) technology to ensure farmer refuge compliance.

FUTURE DIRECTIONS FOR THE BT BRINJAL PARTNERSHIP

Although small in scale, this partnership has been vital in helping Bangladesh move Bt brinjal into farmers' fields where they will obtain the benefits (**Figure 2**). The partnership's role has been, and can continue to be, as a facilitator for the sustainable use of Bt brinjal primarily through capacity building and advising. While BARI is the key stakeholder in the development of the technology in Bangladesh, Bt brinjal technology has other stakeholders to carry forward the technology to the ultimate users (farmers). Other stakeholders include various government sectors (DAE, BADC, etc.), the private sector and NGOs, consumers and the media. The Ministry of Agriculture (MOA) can provide guidelines (policy and logistics) to help the partnership meet the needs of the various stakeholders. Listed below are some comments related to future directions.

a) For the last several years, BARI has produced large quantities of the four Bt brinjal varieties and provided breeder seed to BADC for multiplication. As other varieties are approved,

FIGURE 2 | Bangladesh farmer Md. Saiful Islam and his family harvesting Bt brinjal. Photograph by Md. Arif Hossain and used with permission of photographer and the Islam family.

BARI can likely continue to follow this strategy until the private sector is allowed to enter the market. Meanwhile, the partnership can benefit greatly if BARI continues to focus on varietal maintenance (breeder seed production) and purification (as needed). Other activities can also enhance the partnership in the near and long term. These include: enhanced documentation of an appropriate seed-to-seed stewardship protocol; providing services such as seed testing for presence of the Bt gene to the seed multiplication agencies (currently BADC and maybe the private sector in the future); generating reliable information on Bt brinjal cultivation; communicating such to various stakeholders.

b) BADC is the only public sector institution to have undertaken commercial multiplication and widespread distribution of Bt brinjal seeds in Bangladesh. The partnership believes this is a positive step to help meet the increasing demand for Bt brinjal. But, along with increased seed production, there should be appropriate information provided to farmers about the technology itself and its agronomic and stewardship requirements. BARI is in an ideal position to help meet this critical need.

c) As with any new technology, stewardship is vitally important and this is true of Bt brinjal. Planting borders of non-Bt brinjal as a refuge is critical for the sustainability of the Bt brinjal technology. DAE, as the sole extension arm of the government, is in an excellent position and can play a significant role with technical support from BARI and the project to ensure planting of such refuge crops at the farmers' level. Information about using refuges should be incorporated in extension materials (booklets/leaflets) and training of field staff. Extension material and trainings should also emphasize that Bt brinjal only controls EFSB and that other pests will require supplemental control

d) The private sector has considerable experience developing and bringing GE crops to market while meeting the regulatory requirements of a particular country. In most cases, (except GE papaya in Hawaii), it has been a company that has produced seeds for the GE crop and then sold them to farmers. The private sector (including NGOs involved in commercial seed operations) has not yet but could be a significant partner in the long- term development of Bt brinjal in Bangladesh and future crop innovations. In the seed industry, the private sector is considered to be efficient at developing and scaling quality seed. Once the Bt brinjal technology is made available to the private sector for commercial multiplication, the private sector may readily move forward to develop their own Bt varieties, including hybrids.

e) Since BARI is a public research institution, the MOA remains the ultimate authority to determine the way forward for Bt brinjal at the farmers' level. The technology was approved four years ago, and our partnership is in a good position to work with the MOA to develop a well-defined work plan for full commercialization. A comprehensive work plan with appropriate roles and responsibilities of different stakeholders will help ensure the sustainably of Bt brinjal. The event developer (Mahyco) and other stakeholders in the private sector should be part of this discussion. The role, as well as the responsibility, of the MOA in guiding the way forward of the technology is pivotal. We believe that our project can help facilitate this plan.

THE ROLE OF BT BRINJAL IN BANGLADESH AND THE WORLD

As the first GE crop in Bangladesh, Bt brinjal plays a vital role in the future of biotechnology. The success of this first crop has set the stage for others to come. Fortunately, Bt brinjal has gotten off to a good start with increased yearly adoption and very favorable socioeconomic benefits.

The development and regulation of GE crops in Bangladesh is largely based on agricultural and scientific questions. Their advancement is made possible because the government and people of Bangladesh have embraced science-based technologies that can improve the socioeconomic well-being and environmental safety in their country. Fortunately, the Honorable Agriculture Minister Begum Matia Chowdhury, MP has been essential in making GE crops a reality in Bangladesh. Such support is needed in other parts of the world if the potential benefits of these technologies are to be realized. The success of Bt brinjal in Bangladesh should serve as an example of what can be accomplished with science-based technologies.

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All authors participated in the drafting of this paper as individual subject matter experts in their fields, and the authors are solely responsible for the content. Any views expressed in this paper 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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The Integration of Science and Policy in Regulatory Decision-Making: Observations on Scientific Expert Panels Deliberating GM Crops in Centers of Diversity

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Panels of experts with specialized knowledge and experience are often convened to identify and analyze information relevant for risk assessments of GM crops. A perspective on the use of such scientific expert panels is shared here based on panels convened to inform the regulatory strategy for three separate projects developing GM crops for cultivation in Africa: a nutritionally enhanced sorghum, an insect resistant cowpea, and a virus resistant cassava. The panels were convened specifically to consider the risks associated with gene flow from a genetically modified (GM) crop to naturally occurring 'wild' relatives of that crop. In these cases, the experts used problem formulation to identify effects that regulatory authorities may consider to be harmful ("harms") and formulate plausible scenarios that might lead to them, and the availability of information that could determine the likelihood of the steps in the pathway. These panels and the use of problem formulation worked well to gather the existing information and consider the likelihood of harm from gene flow in centers of diversity. However, one important observation from all of these cases is that it is outside the remit of such scientific expert panels to make decisions dependent on policy, such as which harms should be considered and what information should be considered essential in order for a regulatory authority to make a decision about the acceptable level of risk. These experiences of expert panels to inform GM crop risk assessment demonstrate the challenge of integrating science and policy for effective regulatory decision-making.

Keywords: GM crop regulation, risk assessment, problem formulation, risk characterization, expert panels, gene flow, centers of diversity

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INTRODUCTION

The cultivation of genetically modified (GM) crops can bring significant benefits to farmers, the environment and society (e.g., Klümper and Qaim, 2014; Brookes and Barfoot, 2017a,b). GM crops are also strictly regulated because of concerns that their use may have detrimental effects on human health and the environment (Jaffe, 2004). There is a long-standing concern, however, that 'over-regulation' of the products of biotechnology is preventing the realization of the benefits they offer,

particularly in developing countries (e.g., Paarlberg, 2006). Central to this problem is the need to discern whether and how scientific and non-scientific evidence should be used in regulatory decision-making (Adenle et al., 2018). In particular, assessment of environmental risk is often hindered by the absence of clear policy objectives that are needed to guide the interpretation of scientific data (Evans et al., 2006).

In this perspective, we share observations from our experience with three separate scientific expert panels convened to inform risk assessments on the specific issue of gene flow from GM sorghum, cowpea, and cassava to wild plants in various parts of Sub-Saharan Africa. We found that such panel discussions and the use of problem formulation are first excellent forums for organizing existing knowledge in order to predict the likelihood of harmful effects, at least in these cases of transgene introgression into wild species, including in the crop's centers of diversity; and second, useful for identifying scientific uncertainty associated with the predictions, and studies that could be conducted to reduce that uncertainty. In addition to these, the most notable observation on these expert panels is the need to discern scientific and non-scientific information, as was evident in these discussions. Outside the remit of such panels is decision-making responsibilities that include definitions of harm and judging the sufficiency of scientific knowledge and the extent to which uncertainties must be reduced for decision-making. Hence, the panels demonstrate the challenge of integrating scientific and non-scientific policy-related information in decision-making and the need for clear policy in order to avoid an unnecessary quest for more and more scientific information.

THE PROBLEM: ASSESSING THE RISKS FROM GENE FLOW TO WILD RELATIVES

The potential for harmful effects following gene flow from GM crops to sexually compatible wild relatives was among the earliest environmental concerns associated with GM crops (e.g., Dale, 1992). Frameworks to assess the risks from gene flow to wild relatives are not defined as well as those to assess some other risks, such as the use of substantial equivalence for food safety (e.g., Novak and Haslberger, 2000; König et al., 2004), or the tiered approach that is used for non-target organism assessment (e.g., Garcia-Alonso et al., 2006; Romeis et al., 2008). Early gene flow studies were concerned more with the frequency and distance of gene flow (e.g., Timmons et al., 1995; Ellstrand et al., 1999; Ellstrand, 2003), although these studies rarely lead to a risk conclusion without a need to consider the consequences. It is more difficult and few attempts have been made to design frameworks and studies that assess the more complex questions about consequences of gene flow (although, see Snow et al., 2003; Raybould and Cooper, 2005; Sasu et al., 2010).

The crops which were the subject of the expert panel discussions included sorghum (*Sorghum bicolor*) and cowpea (*Vigna unguiculata* subsp. *unguiculata*), which are staple crops being developed for use in, or close to, their center of diversity in Africa. The center of diversity can be defined as the geographic area where there is a high level of *in situ* genetic diversity for a crop species. The third crop, cassava (*Manihot esculenta*), is a staple crop but its center of diversity is not in Africa; however, there is one known, introduced compatible wild (free-living) relative of cassava (*M. glaziovii*) found in Africa. These three crops are the subject of continuing research to use genetic engineering to introduce traits with the potential to dramatically improve value for farmers and consumers in Africa: sorghum with multiple genes for nutritional enhancement traits (increased vitamin A, iron, zinc, lysine, and threonine) in East and West Africa; cowpea producing Cry1Ab that confers resistance to the pod borer in West Africa; and cassava using RNA interference (RNAi) technology for cassava brown streak virus (CBSV) resistance in East Africa.

The first regulatory scrutiny of these and similar GM crops being developed by international non-profit, philanthropic or governmental development organizations, is likely to occur in countries where regulatory authorities have limited experience of and resources for evaluating the technology, including risk assessment. It is important, therefore, that risk assessments exploit existing knowledge and not default to requirements for new data when it is not necessary for effective decision-making, as is an unfortunate trend in cases where there are more resources. To this end, the first of these panels, comprising experts in risk assessment, gene flow, sorghum biology and sorghum as a crop in Africa was assembled in 2008 by the Africa Biofortified Sorghum project to discuss how to assess the risks from cultivating nutritionally enhanced sorghum in the center of sorghum diversity. Sorghum is a major crop in sub-Saharan Africa and its center of diversity is in Ethiopia and Sudan (Harlan, 1971); therefore, if GM sorghum is to be grown in Africa, the question of risks from gene flow in centers of origin and diversity has to be addressed. It is important to note that the panel was not asked to make a decision about the risks, but to share their experience and expertise within the framework of problem formulation and likelihood assessment.

The sorghum panel provided the template for the composition and method of working for the subsequent panels on cowpea and cassava in East Africa. Each panel selected by the projects consisted of approximately six scientific experts (not regulators), half being experts from Africa, who had expertise in the area of GM crop risk assessment, gene flow, or the biology and ecology of the crop and its relatives. Although other panel members differed among panels, the authors of this perspective participated on each of these panels, and the three discussions were facilitated by author K. Hokanson. The sorghum and cowpea panels met in St. Louis, MO, United States in 2008 and 2010, respectively; the cassava panel met in Mombasa, Kenya in 2015. Each panel more-or-less followed the process of problem formulation as outlined below. More details of these potential products and of the panel discussions are described in previous publications (sorghum: Hokanson et al., 2010;

cowpea: Huesing et al., 2011; and cassava: Hokanson et al., 2016).

PROBLEM FORMULATION AND LIKELIHOOD ASSESSMENT USING EXISTING INFORMATION

Problem formulation for assessing the risks from using GM crops may be regarded as a method for formulating and proposing tests of hypotheses that are relevant for making decisions concerning particular products (Raybould, 2006). At their most conservative, the hypotheses under test are similar for all crops: growing genetically modified crop X in region Y will not result in harmful effect Z. Less conservative hypotheses are that growing the crop poses no unacceptable risk. Corroboration of a hypothesis of no harm provides rigorous corroboration of a hypothesis of no unacceptable risk, whereas falsification of a hypothesis of no harm does not necessarily indicate unacceptable risk – the risk may be acceptable depending on, for example, the opportunities presented by cultivating the crop (Sanvido et al., 2012). Hypotheses of no harm or of no unacceptable risk are called "risk hypotheses."

After problem formulation, the risks can be characterized by testing the risk hypotheses with existing information. 'Testing' a hypothesis does not necessarily require experimentation. If a hypothesis is corroborated or falsified using available existing information with sufficient certainty for decision-making, no further testing of that hypothesis ought to be necessary for the purposes of risk assessment; however, there may be interest in testing the hypothesis for other reasons. If a hypothesis requires further testing for decision-making, problem formulation devises testing through new studies or observations, or by gaining access to previously unavailable existing information. Because risk assessment is a decision-making tool, and not basic research, simple, rigorous tests of hypotheses under unrealistically conservative conditions are generally preferred. If a risk hypothesis is falsified under conservative testing, a further round of problem formulation may lead to a decision to conduct further testing under more realistic conditions, or to complete the risk assessment based on the conservative tests (Raybould, 2006).

The expert panels followed the principles of problem formulation outlined above as a means to gather and deliberate about existing information. First, the panels decided what effects of gene flow from the crop to a wild relative should might be regarded as environmental 'harm' by a regulator. ('Harm' in this sense is synonymous with 'adverse effects' as used in the methodology outlined for risk assessment under Annex III of the Cartagena Protocol on Biosafety.) Determination of which harms are to be considered in regulatory risk assessment is a matter of policy and would normally be derived from protection goals; that is, the overall objectives of the policy that the regulations are intended to deliver (e.g., Hommen et al., 2010; Garcia-Alonso and Raybould, 2014). As the panels were operating independently of any specific policy guidance, determination of harm was based on precedent from existing risk assessments and assumptions about change that might be regarded as detrimental to the environment, and as such represented an opinion of the experts rather than a regulatory determination. For these panel discussions, harms were defined necessarily to carry out the problem formulation; they were certainly not intended to influence the regulatory policy of any country.

The panels identified a similar list of harms to consider further for each of the crops, which can be summarized as arising from two basic mechanisms: (1) genetic changes resulting from selective sweeps or genetic swamping; and (2) demographic changes resulting from changes in species abundance or an increase in toxicity or decrease in nutritional quality of the wild relative (**Figure 1**). The harm due to the first mechanism would be a loss of valuable genetic diversity in the crop gene pool. From the second mechanism, harms included reduced abundance or diversity of valued species (native flora and fauna or domestic animals) or reduced crop yield or quality through loss of ecosystem services. Loss of valuable ecological functions underlying other ecosystem services was also postulated, but more precise harms were not specified. The panels recognized that the presence of transgenes in wild populations in the absence of any other genetic or demographic effects might be considered of concern on religious or cultural grounds; however, these harmful effects were not considered further as science has little or no contribution to characterizing risk in such circumstances other than to indicate whether or not gene flow is conceivable.

Defining the harms allowed the panels' deliberations to concentrate on elaborating the steps that would need to occur for a harm to be realized; the series of steps leading to a particular harmful effect is called a "conceptual model" or "pathway to harm." A highly simplified summary of the two principal pathways considered by the panels is depicted (**Figure 1**). Without this focus on defined harmful effects, it is likely that the panels would have attempted a comprehensive description of all possible effects following release of the particular GM crop, which is neither an efficient nor effective method of risk assessment.

Once the pathways to harm had been described, the likelihood of each step being realized was evaluated by the panels using existing knowledge. A likelihood assessment determines the degree of chance that harm, or a step leading to harm, occurs OGTR (2009). Ascribing a likelihood to a step, e.g., highly unlikely, unlikely, likely, is in effect a determination of the confidence in the corroboration or falsification of the hypothesis that the particular step in the pathway will not occur. In theory, once a single step in the pathway is deemed as highly unlikely with sufficient confidence, the risk via the pathway can be designated as negligible. However, just as harm and acceptable risk are matters for policy, so is the determination that a hypothesis has been corroborated with sufficient rigor. Hence, even though a particular step in a pathway is deemed unlikely, the discussion of subsequent steps usually continues so that risk could be determined as the cumulative probability of every step being realized in sequence. For each crop, the cumulative probability along each pathway suggests that harm was unlikely

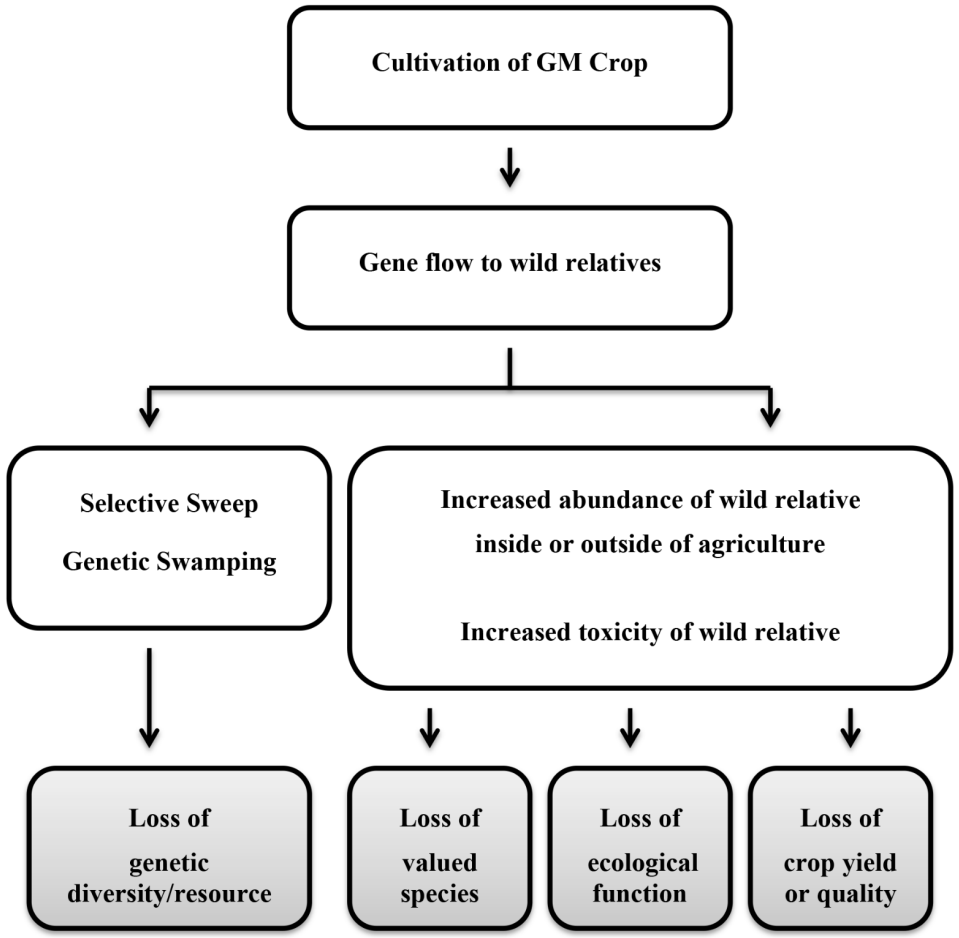

FIGURE 1 | Simplified pathways to harm resulting from gene flow to a wild relative. Gray boxes indicate the harm (adverse effect).

to occur via gene flow from the respective crop to its wild relative (Hokanson et al., 2010, 2016; Huesing et al., 2011).

PROBLEM FORMULATION AND LIKELIHOOD ASSESSMENT IN CENTERS OF DIVERSITY

In these panel discussions, the harmful effects as defined were the same whether or not the crop is grown in its center of diversity (as for sorghum and cowpea) or not (as for cassava), and the process of problem formulation and risk assessment is the same in all cases, although the pathways may be different and more or less probability may have been assigned to different steps. That is to say, different methodology was not necessary to conduct risk assessment for a GM crop in the center of diversity. When conducting risk assessment for GM crops in centers of diversity, the most important thing is to define the harmful effects at the outset. In centers of diversity the primary (although not the only) concern is likely to be the protection of a genetic resource. If this is the case, it is essential that a plausible mechanism by which that harm may arise from growing the specific GM crop is set out, as it was in the panel deliberations.

The harms and the pathways that can lead to their manifestation as defined by the expert panels are a useful start for any discussion of the risks of crop to wild relative gene flow. Particularly in centers of diversity, a loss of diversity in the gene pool of the crop resulting from gene flow has an increased probability than elsewhere simply because compatible wild relatives and a high level of valuable diversity are usually found in a crop's center of diversity. The cassava panel used their knowledge about the center of diversity for cassava to determine that 'loss of genetic diversity' is unlikely if the GM cassava is grown in Africa because valuable genetic diversity in wild relatives is not found in Africa. In the case of sorghum and cowpea, intended to be grown in (or close to) its center of diversity in Africa, it was also important to consider other parts of the conceptual model that would lead to this harm, that being the likelihood of steps for genetic swamping or selective sweeps to occur. The panels agreed, based on their knowledge, that in these cases these steps in the pathway leading to a loss of genetic diversity as a resource for all three crops are also not likely. In

other words, the probability of genetic swamping or selective sweeps is 'no more likely' than with non-GM sorghum, cowpea, or cassava counterparts.

CONSIDERING OPTIONS FOR FURTHER TESTING

After reviewing the estimates of the likelihood of harm based on existing knowledge, in each case, the panels were asked to consider data gaps and ways in which they could be filled. It should be noted that in each case, the panels determined that the risk hypothesis in the first step in the pathway – 'gene flow to wild relatives will not occur' – could be falsified based on existing knowledge. In other words, for each crop there is some evidence to suggest that gene flow can occur between the crop and the wild relative in question. Although the existing quantification of frequency or distance of gene flow was not necessarily precise, the panels thought in each case that it would be low, and additional studies to further test the risk hypothesis, e.g., more precisely measure gene flow, would not usefully reduce uncertainty for the purposes of risk assessment.

Despite ultimately finding that the potential for harm was unlikely via any pathway, based on information considered relevant in all of the steps, each panel suggested options for experimentation to further test hypotheses derived from the pathways. These options are summarized in **Table 1**. These were hypotheses that the panels did not necessarily think 'should be' tested, but that 'could be' tested with a carefully designed experiment, although the panels stopped short of describing details of experimental designs. The relevant project team was left to decide whether to undertake these studies depending on its own priorities, including the *a priori* interpretation of what might be required in the country where the project would apply for an approval.

USING SCIENTIFIC EXPERT PANELS IN RISK ASSESSMENT

Expert panels proved an effective means to allow experts in different disciplines, and sometimes at odds about their initial concepts of risk, to work collegiately to organize existing knowledge into effective risk assessments. Experts in risk assessment could show how to use problem formulation and keep discussions focused on topics essential for risk assessment, while experts on gene flow, crop biology and the ecology of the wild relatives could provide the knowledge necessary to test the hypotheses arising from problem formulation. There was great opportunity for knowledge exchange: local experts could learn risk assessment methods, while risk assessment experts could learn how to integrate local ecological and agronomic knowledge into their conceptual models. Finally, expert panels excel in the ability to suggest options for further work, although this can create problems (see below).

A significant disadvantage in these expert panel deliberations, particularly such as these convened to advise the projects, was the limited input of policy to direct the scientific discussions. First, there is the problem of defining harmful effects. For the purposes of these panel discussions, the scientists on the panels had to define harm based on precedent and inference. This means that the panels may not have considered effects that some future regulator may think are important or which may be defined within specific regulatory guidelines or statutes. Conversely, in setting out what could be considered harmful effects, the opinions of the panels could inadvertently influence

TABLE 1 | Probability for harm related to gene flow from GM sorghum, cowpea, and cassava into wild relatives in Africa based on existing information, and options for further testing of risk hypotheses.

	Nutritionally enhanced sorghum *Sorghum bicolor*	**Insect resistant cowpea *Vigna unguiculata* subsp. *Unguiculata***	**Virus resistant cassava *Manihot esculenta***
Center of diversity	Ethiopia–Sudan region of Africa	West Africa	New World – Mexico/Brazil
'Wild' relative known to hybridize	*Sorghum bicolor* subspecies	*Vigna unguiculata* subsp. *unguiculata* var. *spontanea*	*Manihot glaziovii*
Probability for harm	*Unlikely*	*Unlikely*	*Unlikely*
Option for further testing:			
Proposed Hypothesis	Introgression of transgenes from nutritionally enhanced sorghum will not increase the survival or reproduction of crop x wild relative hybrids above existing levels	An increase in seed production does not result in an increase in the abundance of wild cowpea (in the event that introgression of *Bt* genes protected wild cowpea from insect damage and led to greater seed production)	CBSV does not limit seedling recruitment (populations size) of *Manihot glaziovii* (introgression of virus resistance could not increase the population size of the cassava wild relative)
Proposed Test	Compare fitness-related characters in GM sorghum x wild hybrids and non-GM sorghum x wild hybrids	Observe the number of plants that survive to reproduce in plots sown with increasing seed densities	Determine whether CBSV-infected seedlings of *M. glaziovii* survive at significantly lower rates than non-infected seedlings, even under a high-dose exposure to the virus

regulatory policy in countries where the crops are intended to be grown. Scientific expert panels are also commonly convened by the regulatory authorities. When that is the case, the regulatory policies governing the deliberations should be clear, although this remains a challenge for fledgling regulatory systems such as those in the countries targeted by the three projects discussed here. Even where there are well developed regulatory systems, harms derived from protection goals are not always well defined (Garcia-Alonso and Raybould, 2014).

The more significant challenge encountered by the absence of policy in these types of panel discussions, however, is to know whether or when a hypothesis has been tested with sufficient rigor for decision-making; that is, whether scientific uncertainty is unacceptably high and needs to be reduced. In the absence of such policy guidance, an expert scientific panel can always suggest further studies because no hypothesis can ever be proved and some uncertainty always remains. This problem is perpetuated among research scientists based on a misconception that 'science-based' risk assessment means 'research-based' risk assessment; that is, existing knowledge is never sufficient for decision-making and new studies must always be required. Having seen suggestions for further work, regulators may be reluctant to say that the work is not necessary.

Regulators to whom the remit for decision-making does fall, i.e., those responsible to use the scientific knowledge gathered for the risk assessment in order to make a decision about the acceptable level of risk, should be aware that scientific experts can inadvertently drive regulatory policy toward acquisition of new or 'nice-to-know' data through an emphasis on scientific uncertainty. The aim should be to maximize the use of existing knowledge and only require new data that are necessary or 'need-to-know' in order to reduce uncertainty to a level necessary for decision-making (see also Romeis et al., 2009). The types of complex ecological and evolutionary studies that might be designed to reduce uncertainty about the likelihood of harmful effects from gene flow, such as loss of genetic diversity in centers of diversity, are difficult to execute and are apt to lack the precision that would improve decision-making, even when precise quantitative decision-making criteria are defined by policy. An inclination to exercise excessive precaution risks a waste of scarce resources, and might even stop progress on potentially beneficial projects if the studies proposed are too costly or complex and unworkable. Yet, decision-makers will always face the challenge of balancing precaution with uncertainty. Problem formulation and hypothesis testing are useful tools to find and describe this balance.

CONCLUSION

Our experience working with expert panels to inform GM crop risk assessment in centers of diversity leads us to conclude that panels such as these are valuable for gathering and organizing existing information so that it can be considered in risk assessments of GM crops, and problem formulation is a highly effective tool to facilitate this. However, these panel discussions also demonstrated that, while scientific expertise is essential in order to provide the knowledge necessary for making good decisions, science cannot operate in a policy vacuum. Risk assessors need definitions of harm, otherwise they will be forced into an almost limitless task of trying to characterize every conceivable effect of growing a particular GM crop. Furthermore, without knowledge of how decisions will be made, and in particular how the sufficiency of data will be determined, scientists will always be able to suggest new studies, and less experienced regulators may feel pressure to accept that the studies are necessary. Hence, without policy, science may produce data that are unnecessary for risk assessment and data that are not very interesting for basic research (e.g., Raybould, 2010). Suitable integration of scientific and non-scientific factors will be vital for maintaining functioning regulatory systems, especially in developing countries. With resources of all sorts often being severely limited in developing countries, clear policy is needed to ensure that they are used effectively.

AUTHOR CONTRIBUTIONS

KH, NE, and AR participated in the discussions described here in and wrote the manuscript.

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REVIEW
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The Current Status and Development of Insect-Resistant Genetically Engineered Poplar in China

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Poplar is one of the main afforestation tree species in China, and the use of a single, or only a few, clones with low genetic diversity in poplar plantations has led to increasing problems with insect pests. The use of genetic engineering to cultivate insect-resistant poplar varieties has become a hot topic. Over the past 20 years, there have been remarkable achievements in this area. To date, nearly 22 insect-resistant poplar varieties have been created and approved for small-scale field testing, environmental release, or pilot-scale production. Here, we comprehensively review the development of insect-resistant genetically modified (GM) poplars in China. This review mostly addresses issues surrounding the regulation and commercialization of *Bt* poplar in China, the various insecticidal genes used, the effects of transgenic poplars on insects, toxic protein expression, multigene transformation, the stability of insect resistance, and biosafety. The efficacy of GM poplars for pest control differed among different transgenic poplar clones, larval instars, and insect species. The Bt protein analysis revealed that the expression level of Cry3A was significantly higher than that of Cry1Ac. Temporal and spatial studies of Bt protein showed that its expression varied with the developmental stage and tissue. The inheritance and expression of the exogenous gene were reviewed in transgenic hybrid poplar progeny lines and grafted sections. Biosafety issues, in terms of transgene stability and the effects on soil microorganisms, natural enemies of insects, and arthropod communities are also discussed.

Keywords: transgenic poplar, insect resistance gene, toxin protein expression, multigene transformation, biosafety

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INTRODUCTION

Since the first report on transformation of *Bt* gene into *Populus nigra* was published in 1991, transgenic approaches have been widely used in breeding trees for insect resistance and other environmental stress tolerance in China. China is the only country worldwide with longstanding significant commercial Bt poplar plantations, the area has increased to 450 hm^2 since two Bt transgenic poplars were commercialized in 2001 (Lu and Hu, 2011). This means that the greatest experience of using GM poplar resides in China although studies of various species of insect-resistant plants are ongoing worldwide. We hope that this review will help develop the industry in other countries.

Genetic engineering allows rapid insertion of exogenous insect resistance genes into the plant genome and their expression in the plant. The poplar is a model tree species used in research on

woody plant molecular biology, and genetic engineering of poplar has developed rapidly. In the time since the successful transformation of the poplar clone NC-5339 (*Populus alba* × *P. grandidentata*) with a synthase gene conferring resistance to the herbicide glyphosate in 1987 (Fillatti et al., 1987), additional transformation protocols yielding genetically modified (GM) poplars, including *Agrobacterium*-mediated and biolistic transformation, have been developed. A proteinase-inhibitor gene from potato was used early to confer insect resistance on poplars (McNabb, 1987). An insect toxin-encoding gene (*Bt*) of *Bacillus thuringiensis*, a common soil bacterium, became one of the most widely used genes and was successfully transferred into poplar in 1991 (McCown et al., 1991; Wu and Fan, 1991).

To date, insecticidal genes have been obtained from plants, animals, and microorganisms. Genes encoding proteinase inhibitors, phytolectin, amylase inhibitors, and chitinase, are of plant origin, being part of the natural defenses developed by plants to counter insect attack. Animal sources of insect resistance genes include wasps, spiders, scorpions, and mammals. *B. thuringiensis* (a soil bacterium) is a microbial source of insecticidal toxins. The effectiveness of insect resistance genes varies. Overall, genes from plants and animals tend to not confer prospective effects on poplars: some have hardly any effect (Confalonieri et al., 1998) and others produce low levels of insect resistance (Zhuge et al., 2003; Zhao et al., 2005). However, some have been associated with high-level pest mortality sustained over long periods of time (Leplé et al., 1995; Wu et al., 2000; Delledonne et al., 2001). The *Bt* genes have been the most studied and are the best understood. These genes have been extensively modified via removal of AT-rich regions and addition of tissue-specific promoters. Bt toxin is more toxic than other insecticidal toxins at the same levels (Schuler et al., 1998). The *Bt* gene family constitutes a large reservoir of genes encoding insecticidal proteins. Also, the "CryI and CryIII parasporal crystal proteins" have been extensively studied, targeting Lepidoptera and Coleoptera larvae, respectively (Tian et al., 1993; Bradley et al., 1995; Génissel et al., 2003; Klocko et al., 2013).

The major insect pest species currently causing economic loss and ecological problems in poplar in China are trunk borers and defoliators (insects of the Lepidoptera and Coleoptera). Some species reproduce several times a year. Coleopteran pests are often borers and leaf beetles such as *Anoplophora glabripennis* Motschulsky, *Apriona germari* Hope, and *Plagiodera versicolora* Laicharting. Lepidopteran pests include *Hyphantria cunea* Drury, *Lymantria dispar* Linnaeus, *Apocheima cinerarius* Ershoff, *Malacosoma neustria* Motschulsky, and moth species of the Limacodidae and Notodontidae. Therefore, research on *CryI* and *CryIII* and their transformation into poplar has been funded by the National High Technology Research and Development Program of China ("Program 863"). Gene modification and recombination have yielded efficiently expressed genes. Poplars expressing the CryI or CryIII *d*-endotoxin exhibit high-level resistance to Lepidoptera and Coleoptera pests. Insect lethality is high (up to 100%); the toxins also inhibit larval weight gain, retard development, and reduce insect foraging (Wang et al., 1996; Wang G.Y et al., 2012a; Rao et al., 2000; Zhang et al., 2015).

THE MANAGEMENT AND COMMERCIALIZATION OF INSECT-RESISTANT POPLARS IN CHINA

Research on GM poplars in China commenced in the early 1990s; the first milestone was transformation of the *Bt* gene into *P. nigra* (Wu and Fan, 1991). Research efforts in this area continued focusing on white and black poplars and various hybrids (Zhang B. Y. et al., 2005; Hu et al., 2010). After two decades of study, there have been remarkable achievements in the fields of insecticidal gene transfer, toxin expression and transportation, multigene transformation, insect resistance sustainability and stability, and biosafety. The State Forest Administration (SFA) has established several regulatory frameworks for forest genetic engineering; all research on insect-resistant trees must follow certain procedures in terms of project application and approval, small-scale field testing, environmental release, pilot-scale production, and commercialization, each phase must be evaluated by experts (Lu and Hu, 2011). **Table 1** summarizes the approval process for insect-resistant poplars in China. By 2002, only two poplar clones, *P. nigra* carrying the *Cry1A* Bt-toxin gene and a hybrid white poplar (clone 741) with a fusion of the *Cry1Ac* and *API* genes (the latter encoding the arrowhead proteinase inhibitor from *Sagittaria sagittifolia*) had been approved for commercial production (Lu and Hu, 2006). Presently, nearly 22 insect-resistant poplar varieties have been created and approved for small-scale field testing, environmental release, or pilot-scale production. The ecological safety of transgenic plants has become a hot topic and a major obstacle to the use of transgenic plants. Regulatory issues related to transgenic plants concentrate on health, safety, and environmental risks, and especially on the problems associated with (direct or indirect) human or animal consumption of GM trees and their by-products. However, the results are inconclusive. Poplar is a perennial tree with a long growth cycle. It will be necessary to perform a long-term ecological risk assessment before beginning field trials and commercial production of poplars. Therefore, the Chinese government has adopted a cautious attitude; although many other insect-resistant poplar varieties have been obtained following development of the two above-mentioned varieties, no new commercial trees have been approved.

EFFICACY OF GM POPLARS IN PEST CONTROL

Irrespective of the level of insecticidal proteins in transgenic plant tissue, insect bioassays are the most sensitive method of evaluation. A great deal of work may be summed up as follows.

Tolerances Differ Among Larval Instars

Genetic modification of poplar trees affects pest mortality, feeding, growth, and development. However, the various larval instars of the Lepidoptera or Coleoptera differ in their tolerance to toxin. For example, when *P. versicolora* adults and larvae fed on leaves of eight poplar clones expressing different levels of

TABLE 1 | The administrative approval process of insect-resistant poplars in China.

Gene	Poplar	Small-scale field testing	Environmental release and pilot-scale production	Commercialization
Cry1Ac + API	Poplar 741	1996–1998	1999–2001	2002–2007
Cry1A	*Populus nigra*	1996–1998	1999–2001	2002–
Cry1A	*Populus euramericana* cv. "Robusta 94"	2006–2009	2012–2015	
Cry3A + OC-I + Vgb + SacB + JERF36	*P. euramericana* "Guariento"	2005–2008	2009–2013	
Cry3A	*Populus alba × P. hopeiensis*	2005–2008		
Cry1A	*Populus deltoides × P. simonii*	2005–2008		
Cry1A(b) + spider insecticidal peptide gene	*Populus simonii × P. nigra*	2006–2010		
Cry3A	Poplar 741	2006–2009	2011–2014	
Cry3Aa8	*Populus euramencana* cv. "Nanlin 895"	2009–2012		
Cry1Ac + API	*Populus tomentosa* Carr. Clone BL73	2011–2014		
Cry3A + Cry1Ac + API	Poplar 741	2011–2014		
Cry1Ac + API	*Populus euramericana* cv. "74/76"	2011–2014	2016–2018	
Cry3A + Cry1Ac + BADH	*Populus euramericana* cv. "74/76"	2016–2018		
Cry3A + Cry1Ac + NTHK1	*Populus euramericana* cv. "74/76"	2016–2018		

the Cry3A *d*-endotoxin, the mortality of the 1st, 2nd, and 3rd instar larvae attained 100% after 1–2, 2–3, and 3–4 d, respectively, whereas adult mortality was only 0–15% after 1 d, attained a maximum of 95%, and in some clones was no more than 19% after 4 d (Wang G.Y et al., 2012a; Wang et al., 2012b). When *C. anachoreta* larvae (instars 1–4) fed on leaves expressing Cry1Ac, lethal effects on older larvae (3rd and 4th instars) were not marked (mortality 22.22%), whereas the 1st and 2nd instars were greatly affected (mortality 100%); lethality declined with development. *H. cunea* larvae (instars 1–6) could reach 100% mortality, but there were differences among different instars, the 1st instar larvae died after 3 d, while the 6th instar larvae died after 7 d (Zhang et al., 2015). The larval body-length and head width differed among different larval instars. The mortality rates of different instar larvae vary according to the growth and development of the insects. In general, the later/higher the instar larval stage, the less sensitive is the instar to the toxin, and the longer the larval survival time (Gao et al., 2002; Guo et al., 2004). A GM clone highly toxic to 1st-instar larvae may not necessarily exert the same effect on older larvae. Therefore, it is not enough to evaluate a new insect-resistant plant clone using 1st instars alone; all other instars and adults (such as leaf beetles) must be evaluated also.

Insects Differ in Terms of Toxin Sensitivity

Different insect species, even those in the same class and order, differ in terms of sensitivity to insecticidal proteins. Four Lepidoptera species were fed on *Bt + API*-transgenic poplar leaves; the toxin-sensitivities differed markedly. *Micromelalopha troglodyta* was the most sensitive, followed by *H. cunea*, *Clostera anachoreta*, and *L. dispar* (Gao et al., 2004a). The average rate of lethal effects of the 741 transgenic poplar clones pB29 and pB11 on *C. anachoreta* 1st-instar larvae was 75–95%, *H. cunea* was lethal in >95% of cases in most years (Ren et al., 2017). The poplar 741 clone CC84 expressing the *Cry3A* gene modified the behavior of Coleopteran insects. Feeding tests were conducted on *P. versicolora* and *A. germari*. Larvae of instars 1–3 of *P. versicolora* all died after 4 d of feeding (Wang G.Y et al., 2012a), but no more than 50% of *A. germari* larvae died (Zhen et al., 2007; Niu et al., 2011b). This indicated that leaf beetles were more toxin-sensitive than long-horned beetles. Different insects, even those belonging to the same class and order, appear to have different enzyme systems according to variations in body size. Therefore, they showed differential sensitivity and tolerance to the toxic proteins.

Transgenic Clones Differ in Insect Resistance

Different transgenic poplar clones differ in insect resistance. Three levels are usually recognized: high-level resistance, moderate resistance, and low-level resistance. High-level resistance is associated with major mortality (mortality > 80%) of larvae of all instars, regardless of developmental stage. Moderate resistance is characterized by lower mortality but consistent killing of larvae of all instars. Low-level resistance is associated with moderate or low-level mortality of the first 1–2 instars, but much lower mortality (mortality < 40%) and a longer latency to death of older larvae (Gao et al., 2004b; Zhang et al., 2015). The toxicities of 28 1-year-old greenhouse-grown clones of GM (*Cry1Ac + API*) triploid *P. tomentosa* clones were assessed. Insects (*L. dispar* Linnaeus and *C. anachoreta* Fabricius) fed on fresh leaves. These clones varied in terms of insect resistance: 11 subclones had a mortality > 80%, 7 had a mortality of 60–80%, 10 had a mortality < 50%, and some produced no toxic effects (Yang et al., 2006a). The bioassays were repeated 2 and 6 years after the clones were planted in a protected seedling nursery; the clones still varied in terms of insect resistance, which correlated closely with the data from 1-year-old seedlings (Yuan et al., 2007; Li et al., 2009). In a study including eight *P. euramericana* Neva

clones expressing *Cry1Ac* + *API*, five clones killed all stage 1–4 instar larvae of *C. anachoreta*, and the other three clones killed all 1st–2nd instar larvae, but only 22.22% of instar 3rd and 4th larvae (Zhang et al., 2015).

Numerous factors can cause differences in the expression of the same transformation event. Molecular analyses of hybrid aspen revealed that transgene inactivation was always a consequence of transgene repeats (Kumar and Fladung, 2001). T-DNA repeats (the copy number of the exogenous gene in the host chromosome) influenced transgene expression differentially in different transgenic lines (Fladung and Kumar, 2002). The insert location and gene sequences around the integration site of the exogenous gene could also affect transgene expression (Dong et al., 2015).

Many studies have focused on the selection and utilization of transgenic clones with high resistance. Although this approach can control insect pests effectively, the genetic diversity and stability of the forest will be reduced as a result of using a single high insect-resistant clone (Ren et al., 2018). The recommended insect resistance management strategy involves use of a high dose and refuge strategy to slow the development of resistance to *Bt* plants in the target insect. However, designing mixed afforestation strategies using GM clones with high and medium insect resistance is more conducive to maintaining variety and stability and preventing insect tolerance (Andow and Zwahlen, 2006; Hu et al., 2010; Ren et al., 2018).

MULTIGENE TRANSFORMATION

Most early work featured transformation with single toxin-encoding genes. As reports on the emergence of pest-tolerance and the low toxicities of certain proteins increased in number, multigene transformation (two or more toxin-encoding genes), and combinations of the *Bt* gene with other genes, have become increasingly popular in efforts to create cumulative insecticidal effects by expressing genes differing in terms of mechanism of action or specific binding site. The principal techniques are bivalent or multivalent vector construction, co-transformation, double transformation, use of a gene gun, and hybrid formation. **Table 2** shows multigene transformation of poplar varieties, and the insects used for testing, in China.

Bt Associated With Proteinase Inhibitor-Encoding Genes

Proteinase inhibitors, which are natural insecticidal agents and one of the most abundant types of protein in nature, are found mainly in the storage organs of plants, especially seeds and bulbs (Liu and Xue, 2000). These low-molecular weight peptides/proteins interfere with insect digestion (Abe and Arai, 1991; Joanitti et al., 2006). Many relevant genes have been cloned and the effects of the gene products have been studied in insects in terms of poplar insect resistance (Leplé et al., 1995; Hao et al., 1999; Lin et al., 2002; Zhang et al., 2002; Confalonieri et al., 2003; Zhuge et al., 2003). In addition, combinations of *Bt* with proteinase inhibitor genes have been used to create pest-resistant poplar. *Cry1Ac* + *API* were together transferred into *P. tomentosa* Carr., *P. euramericana* cv. "74/76" poplar 84K (*P. alba* × *P. glandulosa* cv. "84K"), and poplar 741 (*P. alba* L. × *P. davidiana* Dode + *P. simonii* Carr. × *P. tomentosa* Carr.) (Tian et al., 2000; Zheng et al., 2000; Li et al., 2007a,b; Yang et al., 2012). *Cry1Ac* + *CpTI* (the latter gene encoding the cowpea trypsin inhibitor) were expressed in *P. euramericana* cv. "Nanlin 895" via co-transformation (Zhuge et al., 2006). *Cry3A* + *OC-I* (the latter gene encoding the rice cysteine proteinase inhibitor oryzacystatin-I) were transferred into *P. alba* × *P. glandulosa* via *Agrobacterium*-mediated transformation (Zhang B. Y. et al., 2005). All such GM clones were more toxic to young larvae and adults of target insects than poplar clones carrying the single genes. However, *Bt*-targeted insects were more sensitive, indicating that *Bt* played the major role (Li et al., 2000; Zhuge et al., 2003; Yang et al., 2006a).

Bt Associated With a Spider Gene Encoding an Insecticidal Peptide

It is well-known that spider toxin can rapidly paralyze insects and mammals (Bloomquist et al., 1996). A spider insecticidal peptide purified from *Atrax robustus* Simon (Araneae: Hexathelidae) venom at Deakin University (Australia) contains 37 amino acids and kills many agricultural pests without harming mammals (Jiang et al., 1995). The gene encoding the toxin was artificially synthesized in the College of Life Sciences, Beijing University, in co-operation with Deakin University, preserving the amino acid sequence, but using plant-preferred codons. Cotton expressing the gene was toxic to *Helicoverpa armigera* and the growth of surviving insects was remarkably retarded (Jiang et al., 1996).

Work on transformation of the fused spider insecticidal gene and the C-terminal region of the *Cry1A*(*b*) gene into poplar became a priority of the Northeast Forestry University of China. *Populus simonii* × *P. nigra*, *P. davidiana* × *P. bolleana*, and *P. euramericana* 108 were transformed after 2000 (Jiang et al., 2004; Lin et al., 2006; Zuo et al., 2009; Zou et al., 2010). Leaves of *P. simonii* with both genes were fed to larvae of *C. anachoreta* and *L. dispar*. The growth and development of larvae were significantly affected; ecdysis was suppressed, the pupae were deformed, pupation was reduced, and pupal weight fell. Transmission electron microscopy of larval midgut showed that the larvae fed poorly and the midgut was deformed (pathological changes were evident in the columnar and goblet cells); toxicity increased over time (Fan et al., 2006; Cao et al., 2010; Zhao et al., 2010).

The insecticidal mechanisms of spider peptide and Bt protein are different. Theoretically, the combination of these two genes should improve insect resistance. There has been no report on the transformation of a single spider gene in poplar. Tobacco possessing a single spider peptide was toxic to *H. armigera*, with a mortality rate of 30–45% (Jiang et al., 1996). This rate is relatively low compared with that for Bt toxin. Therefore, the insect resistance specificity and environmental safety of the *Bt* and spider peptide combination requires further in-depth study.

The Use of Two *Bt* Genes to Expand Insect Resistance

The *Bt* family contains many variants, and different toxins kill different insects (Tang et al., 2004). For example, the Bt toxins

TABLE 2 | Multigene transformation of poplar varieties, and the insects used for testing, in China.

Gene	Poplar variety	Tested insects	Reference
Cry1Ac + *API*	Poplar 741	*Lymantria dispar* Linnaeus	Zheng et al., 2000
		Clostera anachoreta Fabricius	Tian et al., 2000; Yang et al., 2005
		Micromelalopha troglodyta Graeser	Gao et al., 2004a
		Hyphantria cunea Drury	Gao et al., 2004a,b
	Populus tomentosa	*Clostera anachoreta* Fabricius	Yang et al., 2006a
		Lymantria dispar Linnaeus	
	P. euramericana cv. "74/76"	*Clostera anachoreta* Fabricius	Yang et al., 2012; Zhang et al., 2015
		Hyphantria cunea Drury	
	Populus tomentosa clone 85	*Lymantria dispar* Linnaeus	Li et al., 2007a
	Populus alba × *P. glandulosa* cv. "84K"	*Clostera anachoreta* Fabricius	Zhang B. Y. et al., 2005
Cry1Ac + *CpTI*	*P. euramericana* cv. "Nan-lin895"	*Micromelalopha troglodyta* Graeser	Zhuge et al., 2006; Zhang et al., 2012a
		Clostera anachoreta Fabricius	Zhang et al., 2012b
Cry1A(b) + spider insecticidal peptide gene	*P. davidiana* Dode × *P. bollena* Lauche		Zuo et al., 2009
	P. euramericana 108	*Lymantria dispar* Linnaeus	Zou et al., 2010
	Populus simonii × *P. nigra*	*Lymantria dispar* Linnaeus	Jiang et al., 2004; Lin et al., 2006
		Clostera anachoreta Fabricius	Fan et al., 2006
		Clostera anastomosis linnaeus	Zhao et al., 2010
Cry3A + *OC-I*	*Populus alba* × *P. glandulosa* cv. "84K"	*Anoplophora glabripennis*	Zhang B. Y. et al., 2005
Cry3A + *OC-I* + *SacB* + *vgb* + *JERF36*	*P. euramericana* "Guariento"	*Anoplophora glabripennis*	Wang et al., 2007
Cry1Ac + *Cry3Aa* + *API*	Poplar 741	*Hyphantria cunea* Drury	Wang G.Y et al., 2012a; Wang et al., 2012b
		Plagiodera versicolora Laicharting	
Cry1Ac + *Cry3A*	*P. deltoides* cv. "Juba"	*Hyphantria cunea* Drury	Dong et al., 2015
		Plagiodera versicolora Laicharting	
Cry1Ac + *Cry3A* + *NTHK1*	*P. euramericana* "Neva"	*Hyphantria cunea* Drury	Liu et al., 2016
		Plagiodera versicolora Laicharting	
Cry1Ac + *Cry3A* + *BADH*	*P. euramericana* "Neva"	*Hyphantria cunea* Drury	Yang et al., 2016
		Plagiodera versicolora Laicharting	

most commonly used, Cry1A and Cry3A, kill only Lepidoptera and Coleoptera, respectively. Therefore, combinations of Bt endotoxins in the same plants expand the insect resistance spectra, as validated in many studies (Meenakshi et al., 2011; Wang G.Y et al., 2012a; Jiang et al., 2016). In China, two poplar varieties containing both *Cry1Ac* and *Cry3A* have been obtained. One is the hybrid poplar 741, another is the poplar Juba (*P. deltoides* cv. "Juba"). Ten hybrid poplar 741 clones with two insect resistance genes (*Cry1Ac* + *API*) were first created in 2000 (Tian et al., 2000), and bioassays of four clones (pB29, pB17, pB12, and pB11) used as food for *H. cunea* and *L. dispar* revealed obvious insecticidal effects (Gao et al., 2004b). In 2012, *Cry3A* was transferred into pB29 via *Agrobacterium*-mediated double transformation (Wang et al., 2012b). Compared with poplars expressing *Cry3A* or *Cry1Ac* alone, poplars with *Cry1Ac* + *Cry3A* + *API* were toxic to both Lepidoptera and Coleoptera (Wang G.Y et al., 2012a; Wang et al., 2012b). Dong et al. (2015) constructed a plant expression vector containing both *Cry1Ac* and *Cry3A*, the processes involved a simple, rapid, and efficient genetic transformation technique. The transformation of poplar Juba showed that the genome obtained the two *Bt* genes simultaneously.

Apparently, the combination of two or more *Bt* genes with different insecticidal specificities can expand the insect resistance spectra. There are relatively few poplars with such characters and more research is needed to understand the potential associated benefits and problems.

Bt Associated With Genes That Do Not Induce Insect Resistance

Genes that improve plant stress tolerance, such as drought- and cold-tolerance genes, salinity- and alkalinity-tolerance genes, and other stress-related genes, were cloned earlier and transferred to poplars in China (Yang et al., 2001; Fan et al., 2002; Sun et al., 2002; Zou et al., 2004). The search for new stress-tolerance genes (e.g., *eIF1A*, *DREB1C*, *GmNHX1*, and *OsNHXI*), and modification of the available genes to further improve poplar traits, are ongoing. The aim is to increase tolerance to both biotic and abiotic stresses (Li J. et al., 2010; Huang and Tian, 2011; Sun et al., 2013; Ji, 2015; Zhu and Wang, 2015). Transformation of *Bt*, together with other insect resistance genes, into such plants to confer multiple useful traits is also being studied.

Five cloned genes, a regulatory gene (*JERF*36), a levansucrase-encoding gene (*SacB*), the gene encoding the *Vitreoscilla*

hemoglobin (*vgb*), and the "binary coleopterous insect resistance" gene (*Cry3A* + *OC-I*) were co-transferred into *P. euramericana* "Guariento" using a gene gun. Twenty-five kanamycin-resistant plants were obtained, of which seven contained all five genes as revealed by polymerase chain reaction (PCR) and Southern hybridization (Wang et al., 2007). These plants grew well on coastal saline soil in Dagang, Tianjin. Subsequent research showed that the plants were highly tolerant to drought, waterlogging, and salinity (Li H. et al., 2010; Su et al., 2011; Li et al., 2015).

NTHK1 and betaine aldehyde dehydrogenase gene (*BADH*) are two salt-tolerance-related genes; *NTHK1* is an ethylene receptor gene from tobacco, and is induced by mechanical injury, NaCl, and PEG (Cao et al., 2006). The protein encoded by the *BADH* catalyzes the synthesis of glycinebetaine, an important quaternary ammonium compound produced in response to salt and other osmotic stressors by many organisms (Jia et al., 2002). Multigene plant transformation vectors carrying *Cry1Ac* + *BADH*, *Cry1Ac* + *NTHK1*, *Cry1Ac* + *Cry3A* + *NTHK1* (Du et al., 2014; Liu et al., 2014), and *Cry1Ac* + *Cry3A* + *BADH* (Yang et al., 2012, 2016; Liu et al., 2016) were created at the Hebei Key Laboratory for Tree Genetic Resources and Forest Protection, Hebei province. The genes were successfully transformed into tobacco and various poplars; regenerated plants exhibited both pest-resistance and an increase in salt tolerance compared with controls (Ren et al., 2015).

EXPRESSION AND TRANSPORT OF Bt PROTEINS IN POPLARS

Difference in Expression Levels of Bt Proteins

Measurements of Bt protein levels and the results of insect feeding tests revealed a close positive relationship between protein expression level and pest-resistance; clones expressing high Bt levels exhibited high-level insect resistance (Tian et al., 2000; Dong et al., 2015; Liu et al., 2016). To date, *Cry1Ac* and *Cry3A* of the *Bt* family have been most widely used for genetic transformation of insect-resistant poplars in China. Enzyme-linked immunosorbent assay (ELISA) analysis revealed significant differences in Bt protein levels; that of Cry3A was significantly higher than that of Cry1Ac, independent of the transformation method used (individual transfer of *Cry1Ac* and *Cry3A*) (Tian et al., 2000; Yang et al., 2006a; Wang et al., 2008; Niu et al., 2011b), transformation of *Cry3A* into a plant already expressing *Cry1Ac* (Wang G.Y et al., 2012a), or transfer of *Cry1Ac* and *Cry3A* together (two *Bt* genes were constructed in one vector and then inserted into the poplar genome) (Dong et al., 2015; Zhao et al., 2016; Liu et al., 2016). The toxic protein expression levels of the two *Bt* genes differed significantly between the acquired transgenic clones. The expression level of Cry3A (2.24–13.30 $\mu g\ g^{-1}$ FW) was typically 1,000-fold greater than that of Cry1Ac (16.44–60.32 $ng\ g^{-1}$ FW) (Wang G.Y et al., 2012a). In most of these studies, CaMV35S promoter was employed. Dong et al. (2015) tried to find the differences between different promoters and vectors, in their transformation of poplar Juba, two Bt genes (*Cry1Ac* and *Cry3A*) on vector p71A68Y71 were separately driven by promoters CAMV35S and CoYMV, and a matrix attachment region (MAR) sequence was added to both sides; in contrast, two Bt genes on vector p05A68A71 were driven by the promoter CAMV35S without a MAR sequence structure. The result also showed that Cry3A toxic protein content was much higher than the Cry1Ac toxic protein content in all regenerated lines.

The difference in Bt expression levels may be related to the sequence and direction of exogenous vector genes, the exogenous genes species, or the promoter type (Ren et al., 2015). When two genes were constructed in the same vector, the insertion was adjacent. Because of the high homology of the two genes, interference was possible (because one gene inhibited the other). In addition, the arrangement order of the targeted genes may also affect gene expression. Studies on tobacco showed that, when two *Bt* genes were simultaneously constructed in one transformation vector, the *Bt* gene close to the left boundary of T-DNA was efficiently expressed, regardless of whether it was *Cry3A* or *Cry1Ac*, whereas the gene close to the right boundary was inhibited (Dong et al., 2015). This finding requires further research and validation.

Temporal and Spatial Dynamics of Bt Protein Expression

Temporal and spatial studies of Bt protein expression showed that expression varied by the developmental stage and the tissue. The Cry3A protein levels of twig xylem, and the roots of a 2-year-old poplar 741 clone, increased consistently, whereas the leaf level first decreased and then increased over the growing season. In terms of tree crown layers, the Cry3A protein level in xylem increased from the upper to the lower crown, but the leaf pattern was the opposite (Niu et al., 2011a). The temporal/spatial dynamics of Cry1Ac protein expression were also monitored in a plantation of 6- to 8-year-old trees of a transgenic insect-resistant poplar. Cry1Ac protein content changed in a consistent manner, initially increasing and then decreasing over the growing season (6–10 months) of each year, peaking in August, and then decreasing. The levels of Cry1Ac protein were, in rank order: root system > leaves of short branches > leaves of long branches (Zhang et al., 2016). The expression regularity of Bt protein is closely related to the tree growth pattern of *Bt* poplar, in fast growing season and metabolism tissues, Bt protein expression is high accordingly.

Transport of Bt Protein in Transgenic Poplars and Heredity of Exogenous Genes in Transgenic Hybrid Progeny Lines

Wang and Yang (2010) and Wang L.R. et al. (2012) grafted non-transgenic and transgenic poplar 741 (with the *Cry1Ac* gene) samples as both scions and stocks, and Cry1Ac protein transportation studied using ELISA. The protein was detected in non-transgenic tissue (leaf, phloem, xylem, and pith), being especially high in phloem, indicating that the protein moved from

the stock to the scion of grafted plants, principally through the phloem. The protein moved from the roots (stock) to upper parts of the plants (scions), and vice versa. When leaves of grafted branches of non-transgenic 741 poplars were fed to *C. anachoreta* larvae, insect development was delayed. After 8 years of field growth, the transport and accumulation of the Bt protein (in terms of sites of occurrence, levels, and direction of movement) in grafted adult poplars were studied once more. Although some changes were evident, most toxin was transported and accumulated as found previously (Chen et al., 2016).

In the above study on poplar, ELISA analysis showed that accumulation of Bt protein was highest in phloem tissues, indicating that Bt protein was mainly translocated within the phloem across the graft union (rootstock, scion, and interstock) to the leaves. Reverse transcription-PCR (RT-PCR) showed that mRNA of *Bt* gene was not detected in the branch and leaf of non-transgenic poplar 741 no matter its material was used as scion or stock, which suggested that mRNA of *Bt* gene was not transported between the stock and scion (Wang and Yang, 2010).

BIOSAFETY ASSESSMENT

Genetic modification technology should provide substantial economic and long-term environmental benefits. Over the past 30 years, various GM trees with modified characteristics have been created. The wide application of transgenic technology to tree genetics and breeding has greatly accelerated progress (Häggman et al., 2013). Potential risks such as unwanted gene flow and pleiotropic effects of transferred genes attract increasing public attention (Andow and Zwahlen, 2006; Hoenicka and Fladung, 2006). The safety issues include the effects of transgenic poplars on soil microbes, the natural insect enemies of poplar pests, non-target insects, and arthropods as well as transgene stability and the possible impacts of vertical and horizontal gene transfer.

Field Testing and Stability of Transgenes

Culture and regeneration of transgenic plantlets is performed in the laboratory. Transgenic plants are rapidly micropropagated *in vitro* and seedlings with roots are then planted in experimental fields being subjected to adaptive exercises mimicking the external environment. Many reports on annual crops have shown that transgene expression is less stable than originally thought (Meyer, 1995). Trees are perennials with long life-cycles, raising concerns about the stability of exogenous genes and the long-term field efficacy of transgenic tree growth (Hawkins et al., 2003).

The inheritance and expression of the exogenous Bt gene/protein was studied in transgenic hybrid poplar progeny lines. Hybridization was implemented using non-transgenic poplar 84K as the male parent trees, and transgenic poplar 741 lines showing different insect-resistant ability [high insect resistance (pB11, pB29), moderate insect resistance (pB1, pB17), and no insect resistance (pB6)] were used as the female parent trees. The insect resistance of the hybrid progeny plants was nearly identical to that of the parent plants (Ren et al., 2017). This study indicated that using transgenic poplars as parent plants for sexual hybridization could transfer exogenous genes to progeny, and lead to the cultivation of new insect-resistant species.

Polymerase chain reaction, Southern blotting, and ELISA showed that exogenous genes were stable for many years in field-planted GM poplars (Wang et al., 1996; Lin et al., 2002; Zhang et al., 2004; Yang et al., 2006a). Three poplar 741 (*Cry1Ac* + *API*) plantations exhibiting high-level insect resistance were exposed to *C. anachoreta*, *L. dispar*, *H. cunea*, and *M. troglodyta* over 4 successive years. Insect mortality fluctuated somewhat, but no regular decline was noted (Yang et al., 2005). Within the same year, different lines showed different degrees of resistances to the target pests, while differences within the same lines among years were also seen, which may be related to the climate, environment factors, and target pest status, among other factors (Ren et al., 2017). In the 8- and 10-year-old plantations, the diameter at breast height (DBH) of transgenic poplar 741 was also compared with that of non-transgenic poplar. DBH growth in non-transgenic poplar 741 did not differ significantly from that of transgenic lines. This indicates that the exogenous *Bt* gene had no influence on the growth of *Bt* poplar (Ren et al., 2017).

Compared with non-GM poplars, GM poplars protect their foliage from pest outbreaks in the wild. Transgenic poplar trees (*P. nigra*) expressing the *Cry1Ac* gene were evaluated in the field at the Manas Forest Station of the Xinjiang Uygur Autonomous Region during 1994–1997 and 1997–2001. The average proportion of severely damaged leaves on transgenic trees was 10% whereas leaf damage of control trees in nearby plantations attained 80–90%. The average number of pupae per m^2 of soil at 20-cm depth in the test field declined from 18 to 8 pupae per m^2 from 1994 to 1997, but increased in control plantations. In a field trial conducted in 2005 in Huairou, Beijing, Bt-expressing *P. nigra* exposed to *A. cinerarius* exhibited $\leq$20% foliage damage; the figure for control poplars was up to 90% (Hu et al., 2001, 2007).

The integrity and expression stability of exogenous genes in recipient plants are the primary issues in plant genetic engineering research, and are important factors in the application of genetic engineering technology. Under certain circumstances, after the exogenous gene is inserted into the plant, environmental factors may influence the expression stability of the exogenous gene (Brandle et al., 1995). In the process of gene transformation, changes can occur in the exogenous gene fragment inserted in the host chromosome, which may influence the growth and economic traits of plants. Data from the field plantation of transgenic poplar showed that the exogenous genes were stable in the transgenic trees, and the insect resistance of the transgenic lines did not show a downward trend over time.

Survival and Escape of Agrobacterial Vectors

Genetic transformation is usually mediated via agrobacterial vectors (80% of all GM plants) (Wang and Fang, 1998). Such recombinants could escape into the soil if they survive in GM plants, rendering it possible that plasmids could move among soil microorganisms. When triploid *P. tomentosa* transformed

with *Cry1Ac + API* was examined during subcultivation and after transplantation, residual agrobacteria were detected in 3 of 28 culture flasks of cultivars after 24 months of cultivation. The three strains were transplanted into pots for greenhouse cultivation. Agrobacteria were detected in the soil of one pot after 1 month. Thus, GM plants transformed using agrobacteria should be strictly checked before release, and strains carrying the recombinant agrobacteria must not be released (Yang et al., 2006b).

Effects on Soil Microorganisms

There are two main routes by which proteins released from transgenic plants may enter the soil. One is via pollen or litter, and the other is via root exudates GM poplars were studied in the field in terms of residues, decomposition, and the influence thereof on soil microorganisms. Three groups of microbes (bacteria, fungi, and Actinomycetes) of *P. alba* × *P. glandulosa* trees transformed with *Cry3A* were investigated in the first and second years after transplantation. Analysis of variance (ANOVA) and multiple comparison analyses revealed no significant difference in the levels of soil microorganisms under most poplar lines at the same time points (Hou et al., 2009). In a field of 3-year-old poplars with the *CPTI* gene, no extraneous *CPTI* DNA was detected (Hu et al., 2004). The microbial compositions of soil at depths of 0–20 and 20–40 cm did not differ between the GM poplar and control plantations (Zhang Q. et al., 2005).

The toxin protein was found in the soil of 4-year-old transgenic poplar 741 (*Cry1Ac + API*) test fields, but the levels showed a descending trend, at each step in the rank order: root tissue > root surface soil > rhizosphere soil > surface soil. The Bt toxin protein distribution was not associated with microbial levels (Zhen et al., 2011). Studies of the microbial diversity of 5-year-old transgenic poplar 741 showed that transgenic poplars did not affect the physical and chemical properties of the soil or the soil microbial community structure. However, the microbial community structure was obviously affected by the location and season (Zuo et al., 2018).

The levels of the three groups of microbes in the soil of stands of 7-year-old transgenic *P. nigra* carrying the *Bt* gene did not differ from those of control stands (Hu et al., 2004). One study investigated a 10-year-old plantation of *P. euramericana* "Guariento," land with five transgenic poplars, land with non-transgenic poplars, and land without any plants (NP). The transgenic poplar was found to have no significant adverse effects on the soil microorganism system. Non-transgenic poplars and transgenic poplars can both increase the metabolic activity of rhizosphere soil microbes compared with NP soil (Zhu et al., 2015).

Assessment of the impact of genetically engineered organisms on the rhizosphere microbe population in soil has become a hot topic. Numerous studies have been reported on the effect on soil microorganisms: most conclusions were positive, i.e., they had no obvious affects. The influence of transgenic trees and tree litter on microbial communities is dependent on the field site, season, and method used to assess the community. Due to the functional differences among different insect resistance genes, the impact of the expressed toxin protein on soil microorganisms may also differ. Rhizosphere microbes can perceive variations in plant root exudates (Liu et al., 2003). Future work needs to address the long-term effects of transgenic trees; these effects should not only be compared with those of non-transgenic counterpart, but also with other possible changes in the agroecosystem (Dunfield and Germida, 2004).

Influence on Natural Enemies of Insects

To clarify whether transgenic poplars affect the levels of natural enemies of insects, laboratory feeding experiments were conducted; the ladybird *Harmonia axyridis* (Pallas) was fed in the laboratory on the aphid *Chaitophorus populeti* (Panzer) which had fed on leaves of poplar 741 (with *Cry1Ac + API*) (Yao et al., 2006) and *P. alba* × *P. glandulosa* (with *Cry3A + OC-I*) (Zhang et al., 2009). The results indicated that aphid consumption of transgenic plant food exerted no significant effect on the mortality, body mass, eclosion, sex ratio, or developmental times of the larval and pupal stages of *H. axyridis*. Studies showed that transgenic poplars with target insect resistance have no negative effect on the predatory ladybird. It is possible that the potential transfer of toxic proteins from the transgenic poplar via its aphid prey to the predator does not occur or, if it does, is not toxic to the predator. Further studies must be conducted to verify these results.

The population dynamics of the principal pests and their natural insect enemies in stands of transgenic poplars have also been studied. In *Bt*-transgenic *P. nigra* stands, the variety, number, and parasitic ratios of the natural enemies of insects were higher than those in non-transgenic plantations. Inoculation of insect pupae collected from transgenic, adjacent, and control plantations with the wasp *Chouioia cunea* Yang revealed no significant among-stand difference in wasp eclosion rate or number (Hu et al., 2007).

The target pests *L. dispar* and *Lymantria susinella* were well-controlled by the Cry1Ac + API proteins of transgenic poplars, and the non-target insect *Chaitophorus populialbae* was not affected; the population of this insect remained stable. In poplar stands exhibiting high- and medium-level insect resistance, the levels of *Vulgichneumon leucaniae* Uchida, a natural insect parasite, declined significantly; the parasite possibly moved away because of reduced prey levels. Compared with the control levels, those of the predators *Misumenops tricuspidatus* and *H. axyridis* Pallas fluctuated to some extent (Jiang et al., 2009).

It is clear that the insect-resistant poplar can cause the death of the target insects, which will likely have either a positive or negative influence on their natural enemies. The insect community forms a complex food chain. Studies of the interactions between transgenic poplar and the natural enemies of insects not only provide useful information about the ecological safety assessment but also suggest better methods for future biological control of insects, including target and non-target insects.

Effects on Arthropod Communities

Arthropod communities are important components of tree ecosystems. Large time spans of continuous insect feeding in

Bt poplar plantations have attracted investigation, to determine whether insects have developed resistance/tolerance to various controlling factors, and whether the total pest populations within arthropod communities have changed. Studies are typically conducted during the growing season. Plants were selected randomly according to the size of the experimental plantations. Each tree was divided into three layers (upper, middle, and lower), and each layer was divided into four directions: East, South, West, and North. The arthropod community distribution (vertical and horizontal) was surveyed in detail on the ground and in bark and branches. Species richness, dominance, evenness, diversity, and similarity were calculated and analyzed, using the Berge–Parker index, Shannon–Wiener index, evenness index, Simpson's inverted index, and Bray–Curtis dissimilarity index. The differences between transgenic and non-transgenic poplars were also compared (Gao et al., 2003; Zhang et al., 2011; Guo et al., 2018; Zuo et al., 2018).

Experimental plots of 84K poplar were established in Beijing, China, in 2005, containing 84K poplar clones (BGA-5) with the *Cry3A* gene and non-transgenic 84K poplars as a control (CK). During the 3-year study (2006, 2007, and 2008), 4,956 arthropod individuals were observed in field trials, including 2,552 individuals on CK trees and 2,404 on BGA-5 trees. These arthropods belonged to 10 orders and 41 families, and included three functional guilds, e.g., phytophages, predators, and parasitoids. Arthropods of 37 families were found on CK; Lasiocampidae, Aegeriidae, Coreoidea, and Miridae were absent on CK trees, but were observed on BGA-5 trees. Although the families and individuals within functional arthropod groups observed in both populations were different, the dominant families in each guild were similar. The *Cry3A*-mediated reduction of the target pest (*P. versicolora*) was not associated with any effect on a non-target pest (*C. anachoreta*), and, generally, exhibited no significant negative effect on the poplar arthropod community (Zhang et al., 2011). These results suggest that planting *Bt* poplar generally had no significant negative effect on the poplar arthropod community.

A study in a transgenic poplar 741 (*Cry1Ac* + *API*) experimental forest (3-year-old trees) at a coastal forest farm (Tangshan, China) reported reductions in the numbers of defoliating insects, fewer dominant species, increased insect diversity, and evenness in terms of the insect pest sub-community. In the arthropod communities of transgenic poplar clones (pB1, pB3, pB11, pB17, and pB29), insect resistance was found to have a negative relationship with community, similar to that of control poplars (Gao et al., 2003). In the soil, surface, and shrub-grass layers, the arthropod sub-community structures of the high-insect resistance poplar 741 clone were near-normal within the soil layer. The other arthropod communities (surface and shrub-grass) exhibited higher diversity and uniformity indices and greater stability, but a low dominance concentration index (Gao et al., 2005).

Two-year-old *Bt* poplar 741 clones pB29 (*Cry1Ac* + *API*) and CC84 (*Cry3A*) were planted at the Ninghe nursery (Tianjin, China) in 2011. The arthropod communities in the experimental forest were determined over 4 consecutive years (2012–2015). A total of 21,662 insects from 12 orders were recorded. The arthropod community in transgenic poplar 741 trees was similar in structure and composition to that in control poplar 741 trees. The dominant insect species in all poplars was Hemiptera, followed by Lepidoptera, Homoptera, Araneida, and Diptera, whereas numbers of Mantodea, Neuroptera, Odonata, and Acarina were relatively low. The main poplar pests (primarily Lepidoptera) were significantly inhibited in the transgenic poplars; however, the number of Coleoptera pests was generally low, and the inhibitory effect was not clear (Zuo et al., 2018).

Experimental plots containing poplar 107 (*P. euramericana* "Neva") with *Cry1Ac* + *API* were established in 2013 in the Luannan nursery (Tangshan, China). Four clones were investigated in 2015, using 2-year-old trees. In total, 6,818 insects belonging to 2 classes, 8 orders, 43 families, and 58 species were recorded. The dominant species on *Bt* poplar were in the Lepidoptera, Coleoptera, Hymenoptera, and Diptera families, with Lepidoptera species the most abundant. The number of herbivorous insects was significantly lower in transgenic poplar 107, whereas the number of sucking insects was significantly higher (Guo et al., 2018).

The characteristics and compositions of food webs in the arthropod communities were also studied. Compared with that of the non-transgenic poplar 741, the arthropod community of the GM poplar 741 exhibited more nutritional and species diversities, more net complexity, and more generalized information diversity, associated with a strong capacity to resist exogenous interference and to recover rapidly from disturbance (Yao et al., 2014). Many studies use the Shannon–Wiener diversity index to measure the amount of entropy or information in a system (Béla, 1995). However, this method was criticized for being sensitive to sample size and completeness, or for producing counter-intuitive community ordering in some cases (Butturi-Gomes et al., 2014). Guo (1988) made an improvement on the basis of Shannon diversity index and so-called generalized information diversity index, which can be decomposed into nutritional level diversity, species diversity, and network diversity (each has its calculation formula). These diversity indexes focus on the status and roles of species in food webs. Both simple and complex food webs have their fundamental characteristics, and these basic characteristics can reflect the internal changes in the food web structure.

No insect resistance was found after continuous long-term exposure during the period considered in China at present. One reason for this finding is that insect pests do not necessarily feed only on the transgenic trees but they may migrate to other plants diluting the exposure to Bt toxins. Another possible explanation is that the experimental plantations of *Bt* poplar were planted using a randomized block design and the area of each treatment was relatively small, i.e., the field trial of *Bt* poplar clone BGA-5 and the CK was designed in plots of 100 trees for each treatment (10 rows, 10 columns) with 2.0 m intervals between trees. In the *Bt* poplar 741 experimental forest, 25 strains were planted per plot, with three replicates; plants were spaced 2 m × 4 m apart. *Bt* poplar 107 (four transgenic clones) and the control poplar were planted with

30 strains per plot, with four replicates; plants were spaced 2 m × 5 m apart. These experimental designs reduced exposure of the target pests to Bt toxins. If large plantations of GM poplar are permitted in the future, it is likely that selection pressure will be much higher so that pests are more likely to evolve resistance.

Several strategies may be adopted to manage resistance. Establishing separate shelter or mixed shelter is a typical management strategy. Non-transgenic poplar plantations in or around GM trees can provide a sufficient number of pest-sensitive populations to dilute the resistance genes and delay insect resistance. Another approach is to plant poplar intercrops with crops such as cotton. Increasing the toxin expression levels of insecticidal genes and using multiple genes with different mechanisms can also delay the development of resistance. Using tissue-specific or time-specific insecticidal promoters, exogenous genes can be expressed in specific tissues or during specific tree developmental periods, such as during the insect hazard peak, and would be an ideal strategy for the temporal and spatial expression of Bt toxin. Fully assessing the risks of *Bt* poplars prior to commercialization poses great challenges, requiring multiple studies spanning numerous sites and successive generations of trees to evaluate ecological performance.

The stability of arthropod community characteristics in insect-resistant poplar plantations is an important consideration with respect to the safety of transgenic insect-resistant poplars. The arthropod community is a complex food web. Species diversity and quantity are fundamental parameters of the arthropod community; any variation therein will influence the entire community. Field tests are an obligatory early step toward future commercial deployment of GM trees. Monitoring should be part of the general stewardship and conditions for the release of a GM tree. A program offering stronger and more complete monitoring of insect resistance could provide a reliable basis for the implementation of the proposed strategy. Moreover, the safety of transgenic insect-resistant poplars must be assessed continuously. Further studies are required to obtain more detailed data for the biosafety evaluation of transgenic poplar.

The Effect of an Insect-Resistant Poplar-Cotton Ecosystem on the Structure of the Arthropod Community

In the 22 years that have elapsed since insect-resistant cotton was first commercialized in China (James, 2008), cotton farmers have been afforded the obvious benefits of a reduced need for chemical pesticides, a cleaner environment, increased yield, and greater profit (Qiao, 2015). Poplar-cotton agro-ecosystems are common in China (Meng et al., 2004). According to the ISAAA 2016 report, 3.7 million ha of *Bt* cotton and 5.43 million ha of *Bt* poplar were planted in China in 2015 (James, 2015). As increasing areas of land become devoted to transgenic insect-resistant poplar and cotton, studies examining the effects of transgenic plants on target and non-target insects become increasingly important. The transgenic poplar-cotton ecosystem strongly inhibits insect pests, but has no impact on the structure of the arthropod community. The character index of the community indicated that the structure thereof was better than that of a control poplar-cotton ecosystem. In terms of the abundance of nutritional classes, the transgenic poplar-cotton ecosystem was also better than a non-transgenic ecosystem (Zhang et al., 2015a,b). This study further demonstrates the safety of transgenic plants, although the effects of insect-resistant poplar-cotton ecosystems on the arthropod community require comprehensive study, including continuous monitoring and tracking over the long term.

OUTLOOK

Additional Insect Resistance Genes

Bt genes are the principal genes used to transform poplars. To date, >700 *Cry* gene sequences encoding crystal proteins have been identified (Palma et al., 2014); the encoded proteins kill Lepidoptera, Coleoptera, Diptera, and Hymenoptera in the field (Sanchis, 2011). Other anti-pest genes include those encoding plant protease inhibitors and plant agglutinins, non-*Bt* genes from bacteria (*Serratia entomophila*, *Pseudomonas entomophila*, and *Morganella morganii*), and genes from fungi [*Metarhizium anisopliae* (countering beetles or locusts), *Beauveria bassiana*, and *B. brongniartii*]. However, many insect pests are not susceptible to the products of these genes or are only poorly controlled thereby. Thus, further research is necessary to identify more efficient insect resistance genes.

A completely different approach for forest pests control is to use RNA interference (RNAi). RNAi is a biological process in which RNA molecules inhibit gene expression or translation, by neutralizing targeted mRNA molecules (Hannon, 2002; Kupferschmidt, 2013). The first step of using RNAi should be the selection of appropriate target genes, enzymes, or receptors which may cause abnormal growth, development, or reproductive inhibition of target pests. RNAi technology had been successfully applied in the study of various types of insects in recent years and many suitable target genes have emerged, such as chitin synthesis-related enzymes (chitin synthase and chitinase gene) (Arakane et al., 2005; Chen et al., 2008), hormones and receptors related to growth and development (epidermal hormones receptors EcR and juvenile hormones) (Huang et al., 2013), enzymes that modulate energy metabolism (proton metabolized V-ATPase and cytochrome P450 enzymes that metabolize toxic substances) (Tang et al., 2012; Kotwica-Rolinska et al., 2013), as well as neuromodulation pathways receptor and related enzymes (Agrawal et al., 2013). RNAi-mediated down-regulation of poplar had been studied and proved that RNAi is also functional in poplar (Mryer et al., 2004; Bi et al., 2015).

Improving Multigene Transformation and Expression Systems

Highly effective multigene transformation systems for plants have been developed; vectors simultaneously express several

target genes from a single plasmid (Chung et al., 2005). Many technical problems have been overcome, such as limitations in terms of multi-cloning and restriction enzyme sites, vector capacity, the need for dephosphorylation during construction, and end-filling of sticky ends with dNTPs. These steps are time-consuming, difficult, and tedious. Easy-to-use vector systems for multigene expression remain technically challenging. Conventional approaches for delivering foreign DNA include *Agrobacterium*-mediated and biolistics transformation, both of which result in the random integration of one or more copies of the DNA sequence (Baltes and Voytas, 2015). In recent years, genome editing technologies using sequence-specific nucleases have been developed as effective genetic engineering methods to target DNA at specific locations in plant genome (Fan et al., 2015). Genome editing technology can target to specific genomic sites by providing the means to modify genomes rapidly in a precise and predictable manner (Bortesi and Fischer, 2015). Furthermore, multigene interaction problems in transgenic plants require attention. Multigene transformation systems and their efficient expression in trees, such as the poplar, must be further explored and perfected.

Biosafety Marker Genes and Marker-Free Poplar Transformation

Marker genes encoding antibiotic- or herbicide-resistance are often used to select transformed plant cells or tissues after exogenous gene transformation. Once the GM plant is obtained, the marker gene is both unnecessary and undesirable (Ye et al., 2012), complicating further transformation using the same selectable gene (Scutt et al., 2002). Therefore, selective marker gene elimination (marker knock-out) is of increasing interest. Although genomics has afforded a theoretical basis for the rapid and effective removal of marker genes, the process is not trivial (Ni, 2007). Selectable marker-free techniques are of great interest to those who work in plant biotechnology (Gu et al., 2014; Woo et al., 2015), but marker-free transformation to obtain pest-resistant poplars is in its infancy and more work is required.

Creating New Poplar Varieties Using Gene Editing

Gene-editing, or genome editing developed in recent years is a type of genetic engineering in which DNA is inserted, deleted, labeled, modified, or replaced in the genome of a living organism (Maeder and Gersbach, 2016). To date, there are mainly four classes of sequence-specific nucleases for genome editing: meganucleases, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein (Cas) (Gilles and Averof, 2014; Baltes and Voytas, 2015). They can be used to knock out genes or to introduce designed sequences into the genome with far greater efficiency than traditional genetic engineering strategies (Maeder and Gersbach, 2016).

The latest and most advanced technology is CRISPR–Cas9 genome editing technology which originates from type II CRISPR–Cas systems (Doudna and Charpentier, 2014). Since the introduction of CRISPR–Cas9, genome editing has become widely used in transformable plants for characterizing gene function and improving traits, mainly by inducing mutations through non-homologous end joining of double-stranded breaks generated by CRISPR–Cas9 (Yin et al., 2017). Two endogenous phytoene dehydrogenase (PDS) genes in *P. tomentosa* Carr., *PtoPDS* 1 and *PtoPDS* 2, had been knocked out simultaneously using the CRISPR–Cas9 technology, the result indicated the possibility of introducing mutations in two or more endogenous genes efficiently and obtaining multi-mutant strains of Populus using this system (Fan et al., 2015; Liu et al., 2015). Gene-editing techniques also render it possible to improve the pest-resistance of poplars by strengthening endogenous defenses. Highly expressed genes, or specific promoters, can be inserted into the poplar genome to precisely replace native genes or promoters. Mutant plants exhibiting specific high-level expression of insect resistance genes are thus created. Enhancing the expression of certain substances or increasing insect resistance by replacing promoters in poplar is only a scenario that requires further study and verification.

Responsible Use of Anti-pest Genes

Lmitations in the insect resistance in transgenic plants have attracted the attention of researchers both in China and elsewhere (Bates et al., 2005; Christou et al., 2006; Wang et al., 2012b). Associated risks may be reduced using genetic strategies, refuge strategies, protection of genetic diversity, and barrier and field protection (Gao et al., 2003; Andow and Zwahlen, 2006). Many strategies have been applied to agricultural GM plants, while little work has examined forest trees. The strategy of using multiple genes with different characteristics to improve poplar insect resistance and expand the spectra has been proven effective in practice. The introduced gene must be expressed not only in the short term, but also in the long term. Insect resistance/tolerance has not yet been reported in poplar target species, and transgenic poplar lines showed no decrease in these traits after many years of field planting. The interaction between the transgenic plants and the main insect species is still insufficiently understood. Responsible pest management is a long process requiring continuous accurate monitoring.

AUTHOR CONTRIBUTIONS

MY proposed concept and edited the manuscript. GW and YD collected data and wrote the manuscript. XL, GY, and XY collected data and edited the manuscript.

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Readiness for Environmental Release of Genetically Engineered (GE) Plants in Uganda

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Research and development of genetically engineered (GE) crops in Uganda was initiated in 2003 with the launch of a national agricultural biotechnology center at Kawanda in central Uganda. The country has now approved 17 field experiments for GE plants, which were first established in 2006 with the planting of a banana confined field trial that evaluated performance of plants modified to express resistance to black sigatoka disease. Researchers leading the GE experiments have indicated that some of these GE plants are ready for environmental release that is moving beyond confined field testing toward commercialization. The government of Uganda, over the past two decades, has supported processes to put in place an effective national biosafety framework including establishment of a supportive policy environment; creation of a clear institutional framework for handling applications and issuance of permits; building critical capacity for risk analysis; and providing options for public engagement during decision-making. Uganda is ready to make a biosafety decision regarding environmental release of GE plants based on the level of capacity built, progress with priority GE crop research in the country, and the advancement in biosafety systems. Enactment of a national biosafety law that provides for a coordinated framework for implementation by the relevant regulatory agencies will strengthen the system further. In addition, product developers need to submit applications for biosafety approval for environmental release of GE crops so that mechanisms are tested and improved through practice.

Keywords: biosafety framework, biosafety capacity building, GE crops, food safety assessment, risk analysis, risk assessment

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INTRODUCTION

Uganda is one of Africa's fasted growing economies. The county's GDP is now estimated at USD 25 billion, up from USD 17 billion in 2012/13 fiscal period. One major driver of growth in Uganda, and indeed many countries in sub-Saharan Africa, has been agriculture. In the 2016/17 fiscal period, agriculture grew by only 1.6%, far below a possible 10% rate that is required to sustain food security in a rapidly growing continent (UBOS, 2017). The slow growth of the agriculture sector is attributable to several biotic and abiotic constraints. Chief among these are pests and diseases in major staple and commercial crops. Drought and related climate change effects continue to limit crop and livestock productivity potential.

The country, through the National Biotechnology and Biosafety Policy 2008, identified biotechnology as a strategic tool to address many crop and livestock production challenges. In the crop sub-sector, biotechnology initiatives were developed to manage pests and diseases that cannot be adequately addressed using conventional breeding techniques. In addition, some crops such as banana require biotechnology approaches as conventional breeding is ineffective in sterile hybrids that form the bulk of the cultivated banana. Genetic engineering has been explored to improve banana and other staple crops such as potato, maize, and cassava.

Crop biotechnology has delivered benefits to millions of farmers in both developed and developing countries. To date, about 18 million farmers cultivate GE maize, soybean, cotton, alfalfa, sugar beet, canola, papaya, potato, and apple among others. Recent evidence shows significant benefits to farmers arising from more efficient production and increased productivity (ISAAA, 2016). Following 22 years of global commercial cultivation of GE crops, Uganda can harness opportunities by adapting and adopting key GE crops such as herbicide tolerant and insect protected crops.

GE crops have been commercialized for more than two decades without demonstrated actual harm to human health and environment (Bawa and Anilakumar, 2013). Potential environmental risks considered include increased weediness and invasiveness, effect on non-target organisms, and changes in the farming system or ecosystem that may impact sustainable conservation of biological diversity. So far, occurrence of these risks has been low after commercialization. This is attributed to the fact that not all GE plants are associated with any or all these risks, a risk assessments conducted by relevant regulatory agencies prior to commercial release of GE crops, and risk management after release, where it is necessary. It is important to note that these potential risks are not only associated with GE plants, however, this paper take a position that fit-for-purpose ("*no more than necessary, and not less than would be harmful to health*") risk assessment is necessary for any research and development process.

Uganda is steadily developing its regulatory framework to harness the opportunities from modern biotechnology. Compared to its neighboring countries, Uganda's biosafety framework is more advance than in Rwanda, Burundi, Tanzania, Democratic Republic of Congo and Southern Sudan but lagging behind Kenya and Ethiopia. While a number of milestones have been registered and research has progressed to field experiments, the readiness to deploy biotechnology products (environmental release) is yet to be assessed. This paper assesses the country's efforts toward an effective science-based regulatory regime and subsequently the readiness to commercialize GE crops. Focus is on institutional systems and policy environment. Consumer acceptance of modern biotechnology and related market dynamics are not discussed.

ASSESSMENT OF THE CURRENT BIOSAFETY POLICY ENVIRONMENT AND PRACTICES

A biosafety regulatory framework is necessary to ensure that human health and the environment are protected from possible adverse effects of products of modern biotechnology. The biosafety system also provides a basis for public confidence and for legal certainty for research organizations and private sector (industry). The major components of a functional national biosafety framework (NBF) include: a supportive policy environment; an institutional framework for handling applications and issuance of permits; a system for risk analysis and decision making; and a mechanism for public participation in biosafety decision-making. The government of Uganda, over the past two decades, has supported the processes to enable development of these key elements.

Policy Environment

Uganda actively participated in the negotiation and subsequently ratified the Cartagena protocol on Biosafety in 2001. The country took further steps to provide for the obligations of the Cartagena protocol. An interim biosafety system to regulate modern biotechnology research and development has been adopted in the absence of holistic legislation. Uganda National Council for Science and Technology (UNCST) was designated the Competent National Authority that provides regulatory oversight for GE research and development initiatives. The UNCST Act, 1990 gives it mandate to clear all scientific research and development activities in the country.

As part of efforts to develop a holistic biotechnology and biosafety regulatory and development framework, Uganda adopted the National Biotechnology and Biosafety Policy in 2008. The Policy recognizes GE as a tool that can be used to enhance agricultural productivity, improve food and nutrition security, promote conservation and sustainable use of natural resources, and enhance human and environmental health. The Policy, under Section 5.4 commits the Government of Uganda to develop legislative instruments to regulate modern biotechnology applications.

Institutional Framework

The National Biotechnology and Biosafety Policy (2008) requires establishment of an institutional framework to support the regulatory process and articulate strategies for capacity building, infrastructural development and technology transfer. Uganda has established an interim institutional framework to operationalize the biosafety regulatory system. The current institutional biosafety framework, as described below, comprises of the national competent authority, the national focal point, the national biosafety committee, the inspection mechanism and institutional biosafety committees (**Figure 1**).

The UNCST is the designated national competent authority to supervise and coordinate implementation of biosafety in the country. The competent authority houses the secretariat of the national biosafety committee. Among its functions, the

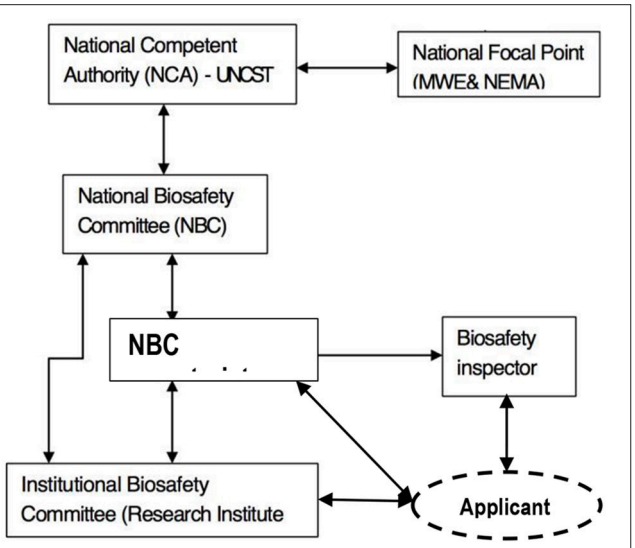

FIGURE 1 | Schematic diagram of the Uganda's biosafety institutional framework.

competent authority approves the development, testing and use of GE products in Uganda, ensures safety of biotechnology to human health and environment during development and testing of GE products and also updates and informs the National Focal Point on matters related to biosafety and biotechnology.

In 1996, the UNCST established the national biosafety committee (NBC) as its technical advisory body for matters concerning biosafety. The main function of the committee is to provide technical advice on biosafety issues to the government particularly with respect to the assessment of benefits and risks associated with modern biotechnology applications and processes. The NBC comprises of relevant experts with competence to review and evaluate risks and benefits of biotechnology research and development activities. The current NBC consists of the following expertise: human health, animal health, plant or animal conservation / biodiversity, biotechnology, social science, agricultural regulation, entomology, legal, environmental chemistry, trade, standard, agriculture, and consumer rights. The NBC can draw upon more experts when necessary.

Institutional biosafety committees (IBCs) have been established in some agencies engaged in biotechnology research and development. IBCs provide a linkage between the NBC and researchers. IBCs are responsible for the initial in-house quality assurance by approving, monitoring, and reviewing contained experiments and recommending confined experiments to the NBC. IBCs also ensure that research by the applicant is done in accordance with conditions of approval set by the NBC. The most active IBC was established by the National Agricultural Research Organization (NARO) in 2004. This IBC has reviewed and overseen more than 20 GE research activities at contained and confined levels.

The Cartagena Protocol on Biosafety requires parties to establish National Focal Points to liaise with the Convention on Biological Diversity (CBD) Secretariat on matters regarding the implementation of the Protocol. The government of Uganda designated the Ministry of Water and Environment as the National Focal Point (NFP) for the Cartagena Protocol on Biosafety. The Competent Authority works closely with the National Focal Point.

UNCST with technical support from development partners like Program for Biosafety Systems (PBS), African Biosafety Network of Expertise (ABNE), and International Center for Genetic Engineering and Biotechnology (ICGEB) has built inspection capacity to oversee and/or enforce regulatory compliance to the terms and conditions of approval. Inspectors were identified from UNCST, the Ministry of Agriculture, Animal Industry and Fisheries (MAAIF), NARO, Uganda National Bureau of Standards (UNBS), universities, Ministry of Water and Environment and the National Environment Management Authority (NEMA). Trained and certified inspectors are designated by UNCST and deployed whenever required.

Capacity for Risk Analysis

Uganda has made tremendous progress in developing human and infrastructural capacity for risk analysis, and biosafety management and enforcement (UNCST, 2016). Currently, there are nine universities that offer biotechnology related courses within a wide scope of other biology-based disciplines. Makerere University, Kyambogo University, Uganda Christian University, and Bugema University in Central region, Busitema University, and Islamic University in Eastern Region, Gulu University in Northern region, and Bishop Stuart University and Mbarara University in Western region. Uganda has also strengthened its biosafety system through short-term training programs for its biosafety practitioners including NBC and IBC members and inspectors.

The country has built more than 10 public biotechnology laboratories, hosted at various universities and research centers. These facilities are capable of conducting basic and advanced biotechnological applications including molecular screening, bioinformatics, plant transformation, tissue culture, and nutrition assays among others. NARO has the most advanced among facilities hosted at Kawanda and Namulonge. About six private agricultural biotechnology institutions are operational, specializing in micro-propagation of coffee, banana, sweet potato, pineapple and potato. There are currently two regulatory focused laboratories addressing GE food safety and GE testing. The existing human and infrastructural capacity can readily be drawn upon for risk analysis, enforcement and management.

Status of GE Research and Development in Uganda

The first application for research using genetic engineering was made in 1992 when Makerere University requested for approval to test bovine somatotropin hormone developed using recombinant DNA technology. Due to limited biosafety capacity at the time, the application was not approved. Plant genetic engineering research in Uganda effectively started in 2003 after H.E. the President of Uganda launched the National Agricultural Biotechnology Center in NARO-Kawanda, central

Uganda. Field experiments testing GE crops were initiated 11 years ago with the planting of the first banana confined field trial that evaluated performance of plants genetically engineered to express resistance to black sigatoka disease. The country has now approved 17 field experiments involving GE crops addressing specific production or nutrition challenges (**Table 1**). The GE crops under testing have been improved for various traits and are at different stages of evaluation.

Banana

Currently NARO is conducting three confined field trials (CFTs) for GE banana at the National Agricultural Research Laboratories (NARL) in Kawanda. Vitamin A rich banana is the most advanced trial as it approaches advanced food safety and nutritional studies. Bacterial wilt resistant banana has been approved for multi-location field testing in two additional sites; south-Western Uganda in Mbarara and Western Uganda in Hoima. Trials are also underway for weevil and nematode resistance at Kawanda.

Cassava

Trials have been conducted for GE virus resistant cassava from 2009. While the first trials focused on resistance to cassava mosaic disease, ongoing regulatory trials in Mubuku, Kasese aim at resistance to brown streak disease. Cassava brown streak disease has now become one the greatest challenges to cassava production in the country, affecting nearly all districts where the crop is cultivated. Annual yield losses are estimated at more than USD 40 million. Progression to regulatory trials under the Virus Resistant Cassava for Africa (VIRCA) project implies the technology has proved effect under field evaluation.

Maize

Uganda experiences occasional moderate to severe drought in selected regions. In the 2016 to 2017 cropping season, severe drought caused significant food insecurity for many families. Drought tolerant maize developed using genetic engineering has been tested for more than 8 years in the country. Trials were initially conducted at Mubuku, Kasese but with the inclusion of stem borer resistance—also developed using GE techniques, other trials were setup in Namulonge, at the National Crops Resources Research Institute (NaCRRI). These research efforts are part of the Water Efficient Maize for Africa (WEMA) Project and build on proven technologies commercialized in other countries. In Uganda, the WEMA trials are among the most advanced toward environmental or general release.

Potato

NARO scientists are evaluating GE potato for resistance to late blight disease at three locations in Uganda. Experiments are underway in south Western Uganda (Kabale), Western Uganda (Kabarole), and Eastern Uganda (Bulambuli). Late blight of potato continues to be a major worldwide threat to potato production and management has been largely through application of fungicides. Integration of late blight resistance into the potato will offer farmers greater flexibility and efficiency in managing this disease.

Soybean

Makerere University, through the College of Agricultural and Environmental Sciences, is currently conducting contained testing of herbicide tolerant soybean. The trials were approved for introgression of proven Roundup Ready technology into locally adapted varieties. Field evaluations will be conducted once stable segregants are identified.

NARO scientists have indicated that many of these research efforts are awaiting an enabling policy environment to move toward environmental release and commercialization. Some technologies are already proven effective in other countries including *Bt* maize and Roundup Ready soybean that have been commercially cultivated for many years. The current experiments in Uganda, including cross-breeding experiments involving proven technologies, imply the need to understand the country's readiness for environmental release of GE crops.

APPRAISAL OF THE ESSENTIAL REQUIREMENTS OF DECISION MAKING FOR ENVIRONMENTAL RELEASE OF GE PLANTS

The process of decision-making regarding environmental release of GE plants is different from that of contained and confined research. While Uganda was able to conduct confined field tests under the provisions of the UNCST Act that governs all STI research, the country took a policy decision that environmental release and commercialization of GE organisms should be guided by an explicit legislative instrument. Biosafety legislation will guide the institutional mechanisms and biosafety decision making systems.

Regulatory Policy

Proposed biosafety legislation was approved by the country's Cabinet in 2012 as the National Biotechnology and Biosafety Bill. The bill provides the scope of regulatory coverage; establishment, description and functions of the decision-making authorities; processes and timelines for different approvals; provisions for conducting risk assessment; socio-economic considerations; monitoring for compliance; and public participation. It also provides for other administrative structures including handling confidential information; enforcement; appeal; fees; labeling; and liability and redress. This proposed legislation was first presented in Parliament in 2013 and was later approved for passage as the National Biosafety Act, 2017. Assent to this law was deferred and the bill is under revision in Uganda's Parliament.

It is important to note that biosafety legislation is not implemented in isolation. Additional considerations for environmental release of GE plants may be provided in other national legislations including: The National Environmental Act (Cap 153); the Plant Protection and Health Act (Cap 31); the Seed and Plant Act (2007); and the Plant Variety Protection Act (2014) (Zawedde et al., 2012).

Uganda has also signed a number of international treaties that may be considered during decision making for environmental release of a GE crop. The Cartagena Protocol on Biosafety

TABLE 1 | Status of GE crop field research in Uganda.

Crop	Trait	Developer/partners	Period of Research	Regulatory status	Estimated commercial release timeline
Banana	Bacterial wilt resistance	NARO; AATF	2010 to date	Multi-location CFTs	2021
	Resistance to black sigatoka	NARO	2006–2009	CFT	–
	Resistance to nematodes	NARO	2012 to date	CFT	2021
	Pro-Vitamin A enhancement	NARO, QUT	2011 to date	CFT	2019
Maize	Drought tolerance	NARO, AATF	2010–2014	CFT	2018
	Insect pest resistance	NARO, AATF	2012–2013	CFT	2018
	Drought tolerance and insect resistance stack	NARO, AATF	2015 to date	Multi-location CFTs	2018
Cassava	Brown streak disease resistance	NARO, Donald Danforth Plant Science Center (DDPSC)	2010 to date	Multi-location CFTs	2019
	Cassava mosaic disease resistance	NARO, DDPSC	2011–2012	CFT	–
Potato	Late blight resistance	NARO, CIP	2015 to date	Multi-location CFTs	2019
Sweet potato	Resistance to viral diseases	NARO	2013–2014	CFT	–
Rice	Nutrient and water efficiency	AATF, NARO	2012 to date	CFT	–
Soybean	Herbicide tolerance	NARO	2016 to date	Contained research	2019
Cotton	Herbicide tolerance and insect resistance	NARO	2007–2012	CFT	–

Source: Research scientists. QUT, Queensland University of Technology; AATF, African Agricultural Technology Foundation; CIP, International Potato Centre.

(CPB, 2000) that requires Uganda, as a Party, to provide for *adequate level of protection for safe transfer, handling, and use of living modified organisms that may have an adverse effect on the conservation and sustainable use of biological diversity, taking into account risks to human health and specifically focusing on transboundary movements*. Uganda under the proposed legislation has adopted the risk assessment guidelines under CPB in the proposed biosafety legislation. The Codex Alimentarius provides a *collection of internationally recognized standards, codes of practice, and guidelines relating to food safety*. Uganda National Bureau of Standards (UNBS) adopted Codex guidelines to develop data interpretation guidelines for use during food safety assessment for GE crops.

Organization for Economic Co-operation and Development (OECD) has *Consensus Documents* that the country may consider to provide *science-based information during environmental risk assessment*. The World Trade Organization treaties including General Agreement on Tariffs & Trade (GATT, 1994), Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement) and the Agreement on Technical Barriers to Trade (TBT Agreement) require Uganda as a Member State to take actions to prevent potential barriers to trade including regulating biotechnology through the adoption of biosafety measures. Decision-making may also be affected by the on-going efforts by the African Union, the Common Market for Eastern and Southern Africa (COMESA), and the East African Commission to harmonize regulation of biotechnology and its products.

For the environmental release of GE crops, Uganda can be guided by implementing provisions in relevant existing national legislation such as the National Environment Act and the Seed and Plant Act while complying with relevant requirements under regional and international obligations. However, this readiness will be greatly enhanced by enactment of an explicit biosafety law that would provide a more coordinated regulatory framework for GE organisms.

Proposed Institutional Framework for Biosafety Regulation in Uganda

A clear institutional framework has been proposed in the new legislation (**Figure 2**). This framework aims to support sound decision-making while building a trusted regulatory system that demonstrates competence, credibility and integrity.

The proposed role of the *Competent Authority* is to link all actors together to ensure safe application of modern biotechnology. The ministry responsible for science and technology will play a policy oversight role as well as act as the national focal point for the Cartagena Protocol for Biosafety. The national focal point role was previously the responsibility of environment ministry. Other relevant ministries, departments and agencies are expected to continue respective mandates of relevance to environmental release of GE crops. The National Environmental Management Authority (NEMA), which is the principal agency in Uganda for the management of the environment is mandated to coordinate, monitor and supervise all activities in the field of the environment. As such, NEMA will play a significant role of participating in the pre-release environmental risk assessment, and in closely monitoring the possible post-release adverse effects of GE plants on conservation and sustainable use of biodiversity. The Government of Uganda is currently at advanced stages of amending the National Environment Act (1995) to among other considerations, codify environmental risk assessment of GE organisms prior to general release.

In addition to the current role of overseeing inspection of research for compliance with phytosanitary measures, the Ministry of Agriculture, Animal Industry and Fisheries through

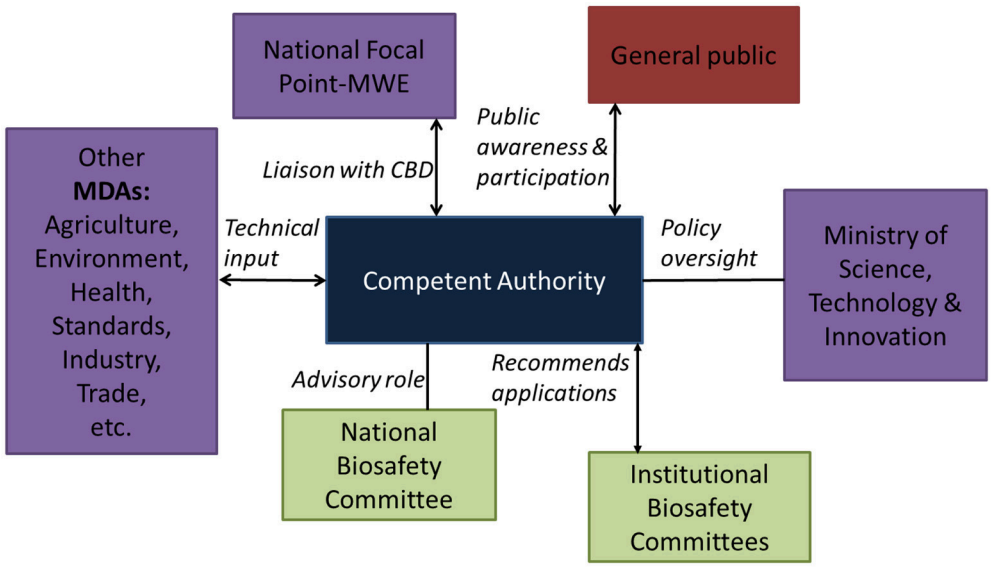

FIGURE 2 | Proposed institutional framework (Under the Bill for National Biosafety Act, 2017).

its Crop Protection department will play a role of regulating import and export of GE organism and regulated agricultural products. Upon approval for environmental release of a GE crop by the competent authority, the agriculture ministry will ensure that variety release procedures are followed prior to commercial release of a GE crop. The Crop Protection department may be delegated by the competent authority to participate in post-release monitoring of the GE plant.

The *Ministry of Health* through its National Drug Authority (NDA) is responsible ensuring the availability of efficacious and cost-effective drugs to the entire population of Uganda. A number of drugs are generated from plants. Plans are underway to use genetic engineering to enhance production of drugs active ingredients in local herbs. It is expected that if some of these trials prove promising and safe, then NDA will play a critical regulatory and safety assessment role prior to approval of the drugs for wider use and application in Uganda. This makes the regulatory agency for drugs in Uganda an important stakeholder in biosafety management.

Ministry of Trade, Industry and Co-operatives is an important player in environmental release of GE plants because it has to advise on socio-economic considerations such as effects on industrial development and on trade. This ministry also provides policy oversight on the *Uganda National Bureau of Standards (UNBS)*. UNBS enforces standards in protection of public health and safety and the environment against dangerous and sub-standard products. The main relevance of UNBS for biosafety is their role in ensuring standards for safety of foods (both locally produced and imported) before they are allowed to be sold or distributed on the Ugandan market.

The proposed institutional framework is inclusive. Its efficiency for environmental release of GE plants will benefit from strengthening the linkages and working relations among relevant ministries, departments and agencies; defining a clear mechanism for compliance enforcement, providing feasible mechanisms for public participation, and building the relevant capacity for risk assessment and risk management within the relevant regulatory agencies.

Capacity for Risk Assessment and Risk Management

Designing and implementing a fit for purpose risk assessment is pertinent for effective risk avoidance, reduction or management. Readiness for environmental release of GE plants requires strengthening necessary capacities for environmental risk assessment and food safety assessment.

Environmental Risk Assessment (ERA)

Environmental risk assessment (ERA) is necessary prior to environmental release of GE plants (Macdonald, 2017). ERA is conducted on a case-by-case basis depending on the GE traits or host plant and the receiving environment. It considers potential risks such as increased weediness and invasiveness, effect on non-target organisms, and changes in the farming system or ecosystem that may impact sustainable conservation of biological diversity. The ERA process involves problem formulation, hazard and exposure evaluation, and risk characterization (Layton et al., 2015). The key factors considered during the ERA process include the host crop that has been improved, the introduced trait and the receiving environment.

Problem formulation involves clear identification of policy requirements and relevant protection goals. Considerations are then made based on existing data and conceptual models to identify biodiversity likely to be exposed, potential harm and exposure pathways (Wolt et al., 2010). Once protection goals and exposure pathways are identified, resources can then be focused on generating missing data necessary for decision making on acceptability of the risk. Hazard and exposure evaluation

is typically based on a tiered approach that commonly uses surrogate species and different exposure levels to increase the efficiency of data collection that may be necessary for ERA (Romeis et al., 2006). A tiered approach is often applied in understanding risks to non-target organisms as identified during the problem formulation stage. During risk characterization, available data is utilized to determine the potential consequences under real environmental conditions. Following the outcomes of the ERA, risk management options will be considered where necessary to mitigate or reduce the level of risks to protect human health and the environment. Typically contingency plans for risk management may be required as part of any conditions imposed during authorization for environmental release.

ERA requires having expertise in key relevant fields such as environmental quality; environmental chemistry; ecotoxicology; environmental risk assessment; microbiology; biochemistry; and handling, monitoring, and remediation of pollution (Soares, 2015). Competences for risk management will stretch beyond technical knowledge to good understanding of procedural aspects of policy making and inspection, and communication with stakeholders. Effective implementation of risk analysis for environmental release of a GE plant will require having a small group of well trained and skilled regulators (Macdonald, 2017).

At national level, most of the required ERA expertise already exist within a number of regulatory institutions, research agencies, universities, and private sector in Uganda. The expertise can be drawn upon to contribute to the risk analysis process necessary for environmental release. At least three scientists working within research and regulatory institutions have received advanced training in ERA, while over 60 scientists and regulators have attended short courses on biosafety risk assessment since 2004.

Uganda's readiness for environmental release of GE plants will require Government investing in strengthening the human capacity within the regulatory agencies by training more risk assessors and risk managers. This may be achieved by conducting tailored, hand-on training programs to strengthen the existing skills, collaborating with experienced risk assessors, and/or through Masters training programs (Komen and Koch, 2017). Strengthening biodiversity conservation capacities will also be necessary.

Food Safety Assessment

GE plants are typically are subjected to food and feed safety assessment that consider potential risks of increased expression of toxic or allergenic compounds, and changes in the nutritional value. In view of the potential impact of biotechnology on the food industrial sector and current research efforts in staple crops, there is a clear need for Uganda to take initiatives to build autonomous capability in food safety assessment and management. Local capability in food safety will be required in selected regulatory agencies such as UNBS, the Government Analytical Laboratory, and food science laboratories in public research and tertiary institutions.

In the recent past, the Program for Biosafety Systems (PBS), African Biosafety Network of Experts (ABNE), and International Center for Genetic Engineering and Biotechnology (ICGEB) have supported building skills of regulators (**Table 2**). The training sessions have focused on building skills to review dossiers for environmental release of GE plants, interpretation of the risk assessment data, and they have worked with some of the relevant agencies to develop standard operating procedures necessary for GE research.

Uganda's readiness will be influenced by willingness to use, and confidence, by our regulatory system in data obtained through outsourcing safety assessments. In such cases, we will need to develop capacity for data transportability and interpretation. Data transportability is the application of data produced in one geographic location to support the safety assessment of that same product in another location (Delaney, 2010). Data generated elsewhere, particularly on food and feed safety, is expected to be useful for similar assessments in Uganda. Data transportability helps to overcome the challenge of allocating resources to carry out comprehensive analytical requirements to establish the "identity" or safety of the proteins in the products. The Uganda National Bureau of Standards has already developed guidelines for data transportability for food safety assessment.

Infrastructural Capacity

Uganda has also progressively built a critical infrastructural capacity (**Table 3**), which can be used to conduct ERA and risk management. This was achieved in collaboration with development partners like USAID, Gates Foundation, Howard Buffet, DFID, Rockefeller, FAO as well as with regional initiatives such as ASARECA, BIOEARN, Biosafe Train, among others. Due to the constant and rapid evolution of this science, the necessary

TABLE 2 | Scientists trained on basic risk assessment for environmental release.

Agency	No. of trained personnel	Existing biosafety competence	Biosafety role
UNCST (includes NBC)	17	Risk assessment; Biosafety reviews; development of guidelines and SOPs	Competent Authority
MAAIF	8	Inspection; risk assessment	Inspections; biosafety support
UNBS	5	Food/feed safety assessment; risk assessment; inspections; development of guidelines	Food safety support
NEMA	5	Inspection; risk assessment	Biosafety support
NARO	15	Risk assessment; dossier preparation; compliance management	Product development; biosafety support (thru IBC)
Makerere University	13	Risk assessment; Inspections	Biosafety support; Training

Adapted with modification from Baguma et al. (2013); personal communications and consists of estimates

level of infrastructural capacity will always be a "moving target" (OECD, 2009). Our readiness will also benefit from Government providing an enabling environment to increase private sector investment in laboratories that can conduct such assessments.

Public Awareness and Participation

Release of GE plants into the environment is of interest to a wide spectrum of the community, including farmers and their associates, government agencies, non-government, and civil society organizations, grassroots communities, media, academia and private sector. Therefore, public awareness is an integral component of every step in regulatory decision-making. Public participation is also critical in the regulatory process for environmental release of GE plants because it allows decision-making to be based on up-to-date and relevant scientific information, and socio-economic considerations for the receiving environment and community (Keese, 2013).

Public awareness efforts to support establishment of a National Biosafety Framework have been on-going since 1996. UNCST, UNEP-GEF, PBS, Uganda Biotechnology and Biosafety Consortium (UBBC), Uganda Biosciences Information Center (UBIC), Science Foundation for Livelihoods and Development (SCIFODE), Open Forum on Agricultural Biotechnology in Africa (OFAB-Uganda Chapter), Tropical Institute of Development Innovations (TRIDI), Cornell Alliance for Science, ISAAA Afri-Center, ABNE and NARO biotech-research projects such as Water Efficient Maize for Africa (WEMA), Virus Resistant Cassava for Africa (VIRCA-Plus), Banana 21 and Banana Bacterial Wilt resistance project have been key players in enhancing public awareness. These efforts have also been focused on enhancing public confidence in the biosafety regulatory system.

In the last 5 years, there has been a lot more public engagement on biosafety focusing on discourse related to the proposed legislation. Trainings were also conducted to empower voices within various stakeholders' groups to support putting in place a functional biosafety system in Uganda. The awareness activities targeted influential champions in various stakeholders' groups including politicians, policy makers, scientists, regulators, media practitioners, extension agents, youth and women groups, and community, opinion, religious, cultural and farmers' group leaders.

TABLE 3 | Laboratory and field infrastructure for GE Plant testing.

Service	Availability in labs and facilities and institutions	Comments
Molecular characterization	NARL, NaCRRI, Government Analytical lab	Staff trained but more equipment needed
Compositional analysis	UNBS, NaCRRI	Mock tests are underway
Full food and feed safety assessment	UNBS (microbial and toxicity studies)	Staff trained in data transportability; mock tests needed
GMO testing	MAAIF, NaCRRI, NARL	Staff trained and equipment available

A recent study conducted by UBIC to assess public knowledge, attitude and perception toward modern biotechnology regulation showed that 65% of the respondents supported having in place a functional biosafety system (**Figure 3**). The study was conducted in 12 districts distributed in all the four regions of the country, and 653 respondents representing various stakeholders participated.

The study further showed that 10% of the respondents did not support having a having a biosafety system because they believed it was synonymous with introduction of GE crops that they oppose. This group together with the respondents (24%) who did not know whether we need a biosafety system are a clear indication of the need for more engagement of key stakeholders relevant to establishment of a functional biosafety system. It is also anticipated that as the country progresses in modern biotechnology application, critics will boost anti-biotech campaigns that may reduce public trust in the biosafety regulatory system. A biosafety communication strategy has been developed by the Ministry for Science, Technology and Innovation to increase public appreciation and confidence in the biosafety system. There is therefore a need to mobilize resources and to strengthen the existing partnerships so that more influential spokespersons are empowered and engaged to support an efficient biosafety regulatory system.

CHALLENGES ASSOCIATED WITH MAINSTREAMING BIOSAFETY IN UGANDA

The government of Uganda has made efforts to mainstream biosafety management, policy development and education through national policies such as Vision 2040 and the National Development Plan II. Further integration of biosafety in regulatory and research agencies is constrained by a number of factors. Existing laws are not explicit on biosafety or regulation

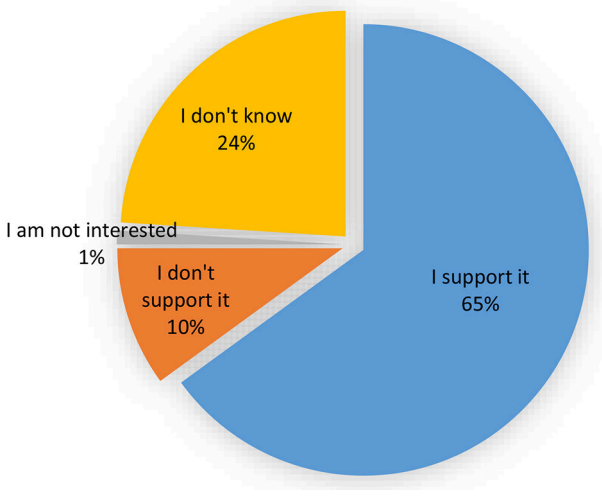

FIGURE 3 | Viewpoints of respondents toward having in place a biosafety system to regulate modern biotechnology.

of GE techniques and products and this can cause conflicting mandates in different regulatory institutions. Most of the existing laws and policies were formulated before Uganda ratified the Cartagena Protocol on Biosafety. Delays in the passage of the national biosafety law is a major set-back in mainstreaming biosafety across sectors.

Another major limitation to mainstreaming biosafety in national systems is the lack of appreciation of the role of an effective biosafety framework in supporting safe advancement of biotechnology applications. This has affected capacity building in various regulatory institutions and research centers. At present, only two institutions have expressed interest in having an institutional biosafety committee. Biosafety awareness building efforts need to focus on key regulatory and research agencies and private sector. Capacity is needed in these agencies for key biosafety areas such as risk assessment and management, GE screening and identification, addressing socio-economic issues, and risk communication.

The high turnover of regulators within key agencies also affects the country's efforts to build an effective biosafety system. While overall capacity exists within the country to regulate many aspects of biosafety noted above, new regulators always require refresher training to understand the issues, best practices, and regulatory procedures. This can be addressed by staggering the appointment of new regulators into NBC, and IBC.

Activism against biotechnology advancement by selected groups in the country has further constrained the mainstreaming of biosafety in national institutions, including the enactment of a biosafety law. As the benefit of a biosafety system is not clearly understood by many leaders, the subject is often associated with advancement of genetically modified organisms (GMOs) that is a divisive subject matter in many developing countries yet the purpose of a biosafety system is regulation.

PROSPECTS FOR ADVANCEMENT OF GE TECHNOLOGY IN UGANDA

Uganda recognized the value of genetic engineering in the late 1990s when it developed a comprehensive poverty eradication plan (PEAP) and plan for modernization of agriculture (PMA) that supported research into and evaluation of GE technologies to address crop production challenges. The country continues to show high level policy interest and action for integration of science and technology in national development. In 2016, the country created a fully-fledged ministry for science, technology and innovation to guide and support advancement of science. The government also established an innovation fund—capitalized initially with about USD 10 million for the first year—to support scientific research and development activities. The new science ministry has been instrumental in leading efforts toward an evidence-based biosafety framework in Uganda.

New initiatives using GE tools under consideration in Uganda may positively influence the ability to make decisions on GE plants. The country, is considering GE mosquito research to address the malaria burden, that costs the country more than USD 100 million to manage each year. National scientists have also collaborated with international private sector partners to test and produce anti-tick vaccines developed through GE technology. The commitment of policy leaders to develop a bio-economy strategy will aid in harnessing some of these tools and products.

The existence of capacity for GE research and capacity for regulation as evidenced by the high numbers of regulators and scientists trained and participating in various aspect of regulation gives greater confidence to stakeholders on the readiness of the country's systems for environmental release of GE plants. Lawmakers had in the past raised issues about the capacity to regulate. Capacity development is nonetheless a continuous effort. Regulatory capacity in Uganda has largely developed in tandem with research progress. This implies that steps have to be taken toward environmental release for the country to build the necessary experience for effective regulation. GE crops approved elsewhere have been proven to be safe using appropriate risk assessment systems. These will form a clear guide for countries such as Uganda where hitherto unreleased GE plants and trait combinations–such as bacterial wilt resistant banana—are being considered.

Opportunities exist to support additional capacity development as may be necessary. A number of national and international initiatives exist that can contribute to these efforts. Some of these initiatives include: African Biosafety Network of Expertise of the New Partnership for Africa's Development (NEPAD); Program for Biosafety Systems (PBS); International Centre for Genetic Engineering and Biotechnology (ICGEB); the International Plant Biotechnology Outreach program of the University of Ghent; Uganda Biotechnology and Biosafety Consortium (UBBC) and Uganda Bioscience Information Centre (UBIC).

Relevant GE crops for Uganda's agriculture have been approved for environmental release in neighboring countries such as GE cotton in Kenya and Ethiopia. This increases the likely for GE crops going through cross border trade and seed exchange. Regional advancements in Ethiopia, Kenya, Malawi and Tanzania toward environmental release of GE crops will build further confidence among Ugandan stakeholders, regulators and policy leaders. As a member of various regional markets such as the East African Community and the Common Market for Eastern and Southern Africa (COMESA), there is opportunity in exploring biotechnology solutions given then improving policy environment within the regional blocks.

ACTIONABLE RECOMMENDATIONS AND CONCLUSION

This review proposes some actionable recommendations for consideration by the relevant ministry and competent authority. The ministry responsible for biosafety needs to strengthen and build new strategic partnerships to support enactment of a national biosafety law and related instruments such as regulation and guidelines. An effective biosafety regulatory system will necessitate the participation and cooperation of other regulatory agencies involved in environmental management,

standards, food safety and plant protection, among others. Operationalization of the law will provide a more coordinated regulatory framework with clear role and responsibilities that will contribute to strengthening the linkages among relevant ministries, departments and agencies.

The competent authority will need to identify, appoint and empower a small group of well-trained and skilled regulators to constitute the NBC. There will be need to develop and maintain a roster of experts that the regulators may call upon to contribute to the risk analysis process. Some of the areas of expertise to be considered include biochemistry; bioremediation; environmental quality; environmental chemistry; ecotoxicology; environmental risk assessment; food science; food safety; microbiology; molecular biology; regulatory enforcement; science communication; science policy among other.

The ministry should also support working relations among the relevant agencies by prescribing mechanisms for good information flow and facilitating periodic networking opportunities. Among these agencies that include NEMA, UNBS, and Ministry of Agriculture, there will be need for capacity building to delineate biosafety considerations from other mandated regulatory considerations of these institutions. Government will need to invest in training more risk assessors and risk managers within these agencies. This may be achieved by conducting short-term to long-term training programs and exchange visits.

The ministry should engage Government to enhance its strategies for attracting science, technology and innovation investment by private sector. An enabling environment together with increased demand from scientists will to increase private sector investment in laboratories that can conduct such assessments.

In some case, outsourcing risk/safety assessment will be the better option. To prepare for such cases that are likely to increasingly become common, the country needs to develop capacity for data transportability and interpretation.

Enhancing awareness, and building confidence, among key stakeholders will require strengthening existing, and building new, partnerships to implement the biosafety communication strategy developed the ministry. Effective communication channels identified by the recent UBIC study will be used to deliver targeted messages to the different stakeholders' groups.

This review also indicates significant progress toward development of key systems necessary for environmental or general release of GE plants. Clear capacity exists for risk assessment. Institutional structures to support approval already exist and will be strengthened by explicit legislation once passed.

As with all GE plants approvals worldwide, a case-by-case consideration will be made by the relevant regulatory system. It is our opinion that Uganda is ready to make a biosafety regulatory decision for environmental release of GE plants based on the level of capacity built, progress with priority GE crops research in the country and advancement in biosafety system.

Enactment of a national biosafety law that provides for a coordinated framework for implementation by the relevant regulatory agencies will strengthen the system further. In addition, product developers need to submit applications for biosafety approval for environmental release of GE crops so that mechanisms are tested and improved through practice.

AUTHOR CONTRIBUTIONS

BZ, identified the essential requirements of decision making and assessed the current status to determine what is needed to enhance the readiness of Uganda for environmental release of GE plants. MK, provided update on the current status of the biosafety and identified challenges associated with mainstreaming biosafety in Uganda. HO, prepared the introduction and provided an update on the current status of GE crop research and development and identified prospects for advancement of GE technology in Uganda.

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REVIEW
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Genetically Engineered Crops: Importance of Diversified Integrated Pest Management for Agricultural Sustainability

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As the global population continues to expand, utilizing an integrated approach to pest management will be critically important for food security, agricultural sustainability, and environmental protection. Genetically engineered (GE) crops that provide protection against insects and diseases, or tolerance to herbicides are important tools that complement a diversified integrated pest management (IPM) plan. However, despite the advantages that GE crops may bring for simplifying the approach and improving efficiency of pest and weed control, there are also challenges for successful implementation and sustainable use. This paper considers how several GE traits, including those that confer protection against insects by expression of proteins from *Bacillus thuringiensis* (Bt), traits that confer tolerance to herbicides, and RNAi-based traits that confer resistance to viral pathogens, can be key elements of a diversified IPM plan for several different crops in both developed and developing countries. Additionally, we highlight the importance of community engagement and extension, strong partnership between industry, regulators and farmers, and education and training programs, for achieving long-term success. By leveraging the experiences gained with these GE crops, understanding the limitations of the technology, and considering the successes and failures of GE traits in IPM plans for different crops and regions, we can improve the sustainability and versatility of IPM plans that incorporate these and future technologies.

Keywords: integrated pest management (IPM), genetically engineered (GE) crops, insect resistance management (IRM), integrated weed management (IWM), adoption of technology, sustainability, extension, genetically modified (GM)

INTRODUCTION

In 1959, the integrated control concept recognized the many ecological and practical advantages of integrating chemical and biological control strategies for pest management (Stern et al., 1959). The concept of Integrated Pest Management (IPM), a corner stone of Integrated Production (IP), appeared in the 1970's, when it became evident that the overuse of chemical pesticides can have serious negative consequences on the environment and human health. The Food and Agriculture

Organization of the United Nations (FAO) defines IPM to be a "careful consideration of all available pest control techniques and subsequent integration of appropriate measures that discourage the development of pest populations and keep pesticides and other interventions to levels that are economically justified and reduce or minimize risks to human health and the environment" (FAO, 2018). Several organizations, including the FAO, the Organization for Economic Co-operation and Development (OECD), and the International Organization for Biological and Integrated Control (IOBC), played a key role in organizing workshops and publishing guidelines related to IPM and IP (Boller et al., 1997, 2004; Wijnands et al., 2012; FAO, 2018; OECD, 2018). IPM is now recognized as a desirable standard for plant protection internationally (e.g., FAO, European Union Directive 2009/128/EC, US Food Quality Protection Act of 1996).

The foundation of an IPM approach is the use of indirect (preventive) crop protection practices, which rely on an understanding of the environment, crop, pest and natural enemy biology, and use of optimized farming practices to manage pests. This includes selection of appropriate crop cultivars for the region, management of soil, nutrients, and water, utilization of sustainable pest suppression practices, as well as implementation of practices that foster the abundance and diversity of beneficial species, such as natural enemies, decomposers, and pollinators. As part of the IPM approach, key pests are closely monitored, and defined intervention thresholds for pest damage or presence are used to indicate when a direct (responsive) crop protection practice is warranted. When required to supplement the preventive practices, the consideration and integration of a broadly diversified set of biological, biotechnical and physical control tactics (e.g., release of natural enemies, pheromone traps or release of sterile insects, and utilization of nets or tillage, respectively) are key to formulation of a diversified, durable, yet flexible IPM strategy that meets social requirements for economic, environmental and human health protection. When pesticides need to be applied, products that are selective are preferred over broad spectrum pesticides. In addition, it is recommended that pesticides are applied with appropriate equipment, optimal dosage, and best timing (Boller et al., 2004; Ervin and Jussaume, 2014; Owen, 2016).

Host plant resistance, whether developed through conventional breeding or through genetic engineering (GE), is a cornerstone of IPM and is a complementary tool to other pest management practices. GE crops have been grown on increasing areas since 1996, reaching 190 million hectares in 2016 globally (ISAAA, 2017). Most GE crops provide tolerance to herbicides (e.g., glyphosate, glufosinate-ammonium, dicamba, or 2-4 D), protection against lepidopteran and/or coleopteran pests, or a combination of both traits. For example, herbicide tolerance (HT) traits that confer glyphosate resistance are available in soybean, maize, canola, cotton, sugar beet and alfalfa, while insect protection, which to date has predominantly been conferred by insecticidal proteins derived from *Bacillus thuringiensis* (Bt), is available in cotton, soybean (lepidopteran pests), and maize (lepidopteran and coleopteran pests). Eggplant in Bangladesh has also contained a Bt trait for a lepidopteran pest since 2014. Additional HT traits that provide tolerance to other herbicidal active ingredients (e.g., isoxaflutole) and other insect active traits (using RNAi, other non-Bt insecticidal proteins, etc.) are being developed to expand the portfolio of GE crops (ISAAA, 2019).

While GE crops may offer additional tools to complement IPM programs and improve their sustainability, economics, and social factors (for example, how one grower's pest management decisions affect surrounding growers and community; Ervin and Jussaume, 2014; Ervin and Frisvold, 2016), an understanding of the characteristics of the crop, the introduced GE trait(s), the crop production system, and the socioeconomic context is critical to successfully integrating GE crops into IPM systems (Meissle, 2016). Current developments in IPM, insect resistance management (IRM) and managing herbicide-resistant weeds were highlighted in a recent symposium organized within the 14th International Symposium on the Biosafety of Genetically Modified Organisms, in Guadalajara, Mexico. Over a series of presentations and a panel discussion, the principles of IPM, the role of socio-economic factors, comprehensive extension to grower communities, and regulations in IPM adoption, and the benefits of using GE crops in an integrated system to improve sustainability were discussed. We present in this paper several case studies where GE crops have been used to manage insects, weeds and diseases and, using these case studies, we highlight the opportunities and challenges for successfully integrating GE crops into an IPM approach in both developed and developing countries. Our examples include GE crops and traits where experience has been gained over many years (e.g., Bt crops, HT crops), new GE plants that have just entered commercial production (Bt eggplant), and GE plants that have not yet been planted commercially (virus resistant common bean).

OPPORTUNITIES AND CHALLENGES FOR USING BT CROPS IN IPM

Over the past 30 years, traits have progressed from single events with one mode of action against one insect order, to pyramided and stacked events containing multiple modes of action against the same or different pest orders, respectively. GE crops have also progressed from insect protection traits expressing proteins from Bt to new traits based on RNAi or expressing proteins from non-Bt sources (ISAAA, 2019). There are many widely accepted benefits of using GE crops for insect control, including the ability to reduce the use of less effective and/or less environmentally friendly insecticides, high specificity toward pests, and a more convenient insect pest management strategy for growers (Brookes and Barfoot, 2013, 2016). An additional benefit seen in some systems, such as with Bt maize in the US (Hutchison et al., 2010; Dively et al., 2018) and Bt cotton in China (Wu et al., 2008) and the US (Carrière et al., 2003), has been area-wide suppression of key target pests that has reduced pest pressure and input costs for both growers adopting Bt crops and non-adopters in the same area. Nevertheless, there remain several challenges for sustainable use of this technology and successful implementation in an IPM approach for many Bt crops and regions.

One of the biggest challenges for sustainable use of the technology is the evolution of resistance. Over-reliance on Bt crops without appropriate IRM or IPM practices has led to a growing number of cases of target pest resistance (Gassmann et al., 2014; Tabashnik and Carrière, 2017). Examples include field-evolved resistance to Cry1Ab-expressing maize in the African stalk borer, *Busseola fusca* (Fuller) (Lep.: Noctuidae), in South Africa (Van Rensburg, 2007); resistance to Cry1F-expressing maize in the fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Fuller) (Lep.: Noctuidae), in Puerto Rico, Brazil and Argentina, and the mainland US (Storer et al., 2010; Farias et al., 2014; Huang et al., 2014); resistance to Cry1Ac-expressing cotton in the pink bollworm, *Pectinophora gossypiella* (Saunders) (Lep.: Gelechiidae), in India (Dhurua and Gujar, 2011); and resistance to Cry3Bb1-expressing maize in the western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Col.: Chrysomelidae), in the US (Gassmann et al., 2011, 2014).

To address the risk of insect resistance, IRM programs have been proactively implemented wherever Bt crops have been commercialized, with these programs being mandatory in some countries including the USA, Canada, Australia, the EU, the Philippines and South Africa (Matten et al., 2008). Central to these IRM programs is the concept of a "refuge," which is an area of plants (typically of the crop of interest) that do not contain any Bt protein and thereby support the production of Bt-susceptible insects (Gould, 1998; Gould et al., 2016). Refuges represent a short-term cost to growers because they incur greater pest damage and require additional management, and thus refuge adoption by growers is generally much higher in countries where IRM is a regulatory requirement e.g., Australia, Canada and the US. The Australian cotton industry represents one success story for adoption of IRM. In the 1990s, Australian cotton-growers faced near catastrophic levels of Lepidoptera resistance to insecticides, which almost led to the end of the cotton industry (Roush, 1998; Roush et al., 1998; Fitt, 2003; Wilson et al., 2018). High awareness of the need for IRM by growers, the availability of different refuge options, and appropriate education and training has resulted in refuge adoption that is consistently near 100% in Australia. Similarly, intensive education together with auditing of growers have helped to maintain high levels of refuge adoption in other countries like Canada (91%) [Canadian Corn Pest Coalition (CCPC), 2018] and, to a lesser extent, the US Corn Belt (68–72%) [Agricultural Biotechnology Stewardship Technical Committee (ABSTC), 2016]. In areas where IRM is not a requirement, disincentives are very high, or growers are not as aware of the costs of resistance, it remains a challenge to educate growers, demonstrate the long-term value of the refuge strategy, and identify other tools to balance the short-term costs. The absence of robust IRM programs can have major consequences; for example, in all the cases of field-evolved resistance described above, one of the primary causes was determined to be low refuge compliance (Tabashnik et al., 2013). Examples of countries where IRM management programs are not mandated include Argentina, Brazil, and China (Wu, 2007; Choudhary and Gaur, 2008). In addition to the lack of refuge compliance, other factors contributing to the evolution of resistance include less-than-high-dose technologies and diverse pest complexes. Overall, regulating IRM and integrating GE crops within the context of a larger IPM plan can help to ensure success, particularly with technologies that are not high dose, but will not be sufficient to do so without extension that leads to broad stakeholder support. Demonstrating the value of IRM within the context of IPM, for example showcasing how GE crops and refugia can better support populations of natural enemies (Lu et al., 2012), or positioning IPM strategies as solutions to greater pest damage in refuges and for non-adopters of GE crops, are important benefits to highlight to promote an integrated approach. For example, insect predator and aphid populations in Bt cotton fields in northern China were assessed over 20 years, from 1990 to 2010, to test the hypothesis that Bt crops can promote biocontrol services at a landscape level (Lu et al., 2012). Results from this study showed that Bt cotton fields with reduced insecticide application supported higher predator populations and decreased aphid abundance. This work supports the hypothesis that widespread adoption of Bt cotton may promote landscape level benefits due to increased generalist predator abundance, and reinforces how IPM strategies that utilize Bt crops and reducing insecticide application can achieve more effective biological control (Romeis et al., 2018).

An additional challenge associated with Bt crops can result if there is a pest shift (i.e., increased prominence of a secondary pest that was collaterally or incidentally controlled by broad-spectrum insecticides but is not controlled by the selective GE trait). For example, in China, widespread adoption of Bt cotton, and the associated decreased use of chemical insecticides, has led to increased abundance of mirid bugs (Hemiptera: Miridae) in some fields (Lu et al., 2010). Any time a primary pest is significantly reduced or eliminated by a technology including a GE trait, there exists the possibility that replacement inputs or other ecological factors will result in a pest shift that may require additional crop protection inputs. If those additional inputs are selective, the overall gains made by growers may still be very positive and IPM is strengthened (Naranjo and Ellsworth, 2009a,b; Ellsworth et al., 2017). However, when new inputs are broad-spectrum, the benefits of adopting the GE trait could be significantly diminished both because of the new input costs and lost opportunities for environmental and human health benefits. A well-structured IPM approach should balance the use of one technology with other complementary approaches and avoid relying on only one solution for pest control. Genetic engineering is not a "silver bullet" for all problems and an agricultural production system will not automatically become a durable IPM strategy just by adding GE technology or, for that matter, host plant resistance developed through conventional means. Therefore, understanding the challenges for each crop, pest complex and region and acknowledging the limitations of GE crops is important for education, training and development of robust IPM strategies for future crops and traits.

IPM OF COTTON IN ARIZONA AND MEXICO

Cotton production in the desert Southwest U.S. has been historically challenged by the presence of several key insect pests

and a wide array of secondary pests. By the early 1990s, the boll weevil, *Anthonomus grandis* Boheman (Col.: Curculionidae), had been successfully eradicated from Arizona through a combination of areawide cultural and chemical practices. At about the same time, the invasive whitefly, a cryptospecies of *Bemisia tabaci* (Gennadius) (Hem.: Aleyrodidae) [= *B. argentifolii* Bellows and Perring], arrived in southern California and Arizona with devastating consequences and established as a key pest of cotton, vegetables and melons thereafter (Ellsworth and Martinez-Carrillo, 2001). This leaf-sucking pest remains today as the number one threat to cotton quality due to their deposits of copious sugary excrement on fiber (Ellsworth et al., 2017). A mirid bug, *Lygus hesperus* (Knight) (Hem.: Miridae), feeds directly on reproductive structures (especially buds and flowers), reducing the number of fruiting sites on the plant and threatening yield production. Another key pest is a boll attacking lepidopteran, the pink bollworm *P. gossypiella*, which is challenging to control because of its cryptic feeding habits inside bolls.

During the first half of the 1990s, insect pest management was dependent on the routine deployment of broad-spectrum chemical controls, such as organophosphates, carbamates and cyclodienes, and pyrethroid mixtures. Resistance and costly secondary outbreaks with mites (Acari), aphids (Hem.: Aphididae), saltmarsh caterpillars [*Estigmene acrea* (Drury), Lep.: Erebidae], cotton leaf perforators (*Bucculatrix thurberiella* Busck, Lep.: Bucculatricidae) or cabbage loopers [*Trichoplusia ni* (Hübner), Lep.: Noctuidae] were common. Foliar spray use was intensive with statewide averages of 10–13 sprays per season (Naranjo and Ellsworth, 2009b).

The introduction of Cry1Ac-containing Bt cotton varieties in 1996 helped to usher in a new era of selective pest control. This trait effectively conferred immunity in cotton to the pink bollworm. Coincidentally, that same year saw the introduction of two selective insect growth regulators (IGRs) for the control of whiteflies. Immediate reductions in foliar insecticide use resulted, though control of *Lygus* bugs still required broad-spectrum insecticides. The success of the GE cotton cultivars was marked by exceptional adoption rates, peaking in 2008 with more than 98% of acreage in Bt cotton after the initiation of a grower-organized pink bollworm eradication campaign (Naranjo and Ellsworth, 2010; Tabashnik et al., 2010). With the introduction of flonicamid, a feeding inhibitor, as the first selective chemical control of *Lygus* bugs in 2006, growers had opportunities to manage all three key pests without the use of broad-spectrum chemistries. The result was an increased role for conservation biological control and a step-change reduction in the use of foliar insecticide (Naranjo and Ellsworth, 2009a; Naranjo et al., 2015; Ellsworth et al., 2017). Starting in 2006 and continuing to this day, Arizona cotton growers spray insecticides, on average, 2.0 ± 0.2 times for all arthropod pests with virtually no sprays for lepidopterans. And, the vast majority of Lygus and whitefly sprays are made with beneficial friendly, fully selective insecticides (Ellsworth, personal communication).

With each new technological innovation (i.e., Bt cotton, selective whitefly IGRs, *Lygus* feeding inhibitor), there was a concomitant need for extensive education, outreach, and extension to growers and their pest managers. Innovations span a continuum of hard technologies (typically complete products like improved seeds, traits, chemicals) to soft technologies (knowledge-based, human-mediated techniques like how to sample and implement thresholds, and IPM strategies). Broad extension support provided by the U.S. Cooperative Extension System is organized federally, within states, and locally within counties to educate, train and facilitate technology transfer to stakeholders. While hard technologies are often technically easy-to-use, their proper deployment depends on accompanying soft technologies that include important translational research and extension adaptation and implementation on a local scale.

The success of the Arizona cotton IPM strategy with Bt cotton as the cornerstone building block of the management system (Ellsworth and Martinez-Carrillo, 2001) was not possible without the significant, ongoing, and progressive inputs from extension as an organized force of mission-oriented research and engaged outreach. Working with Mexican cotton growers immediately across the U.S. border from Arizona and California provided a unique opportunity to examine a counterfactual in a very similar ecoregion and production environment, and largest cotton production region of that country. Their access to most of the hard technologies was contemporaneous to when Arizona cotton growers were adopting them, but there was no analog to Cooperative Extension in Mexico. While Bt cotton was adopted in this region of Mexico at a relatively high rate, growers were still spraying many more times than their Arizona counterparts and exclusively with broad-spectrum insecticides (e.g., methamidophos and many other organophosphates, endosulfan, pyrethroids). Funded by a 17-month grant from US-EPA, an Arizona team conducted an intensive extension campaign in Mexico including grower education, workshops, seminars, demonstrations, and grower participatory trials and validation research (Ellsworth, personal communication). As a result, in 2012 alone, growers decreased their spraying by 31–40%, their insecticide costs by 34% and reduced the use of broad-spectrum insecticides by 23–86% for a savings of over $1.6 million. This lends support to the conclusion that GE crops like Bt cotton or any other hard technology are very dependent on the set of adaptive research and strategic solutions that constitute soft technologies (especially IPM), and further that Cooperative Extension or an analog is key to the transfer of both hard and soft technologies simultaneously.

The Arizona cotton IPM strategy has cumulatively saved growers over $500 million since 1996 in yield protection and control costs ($274/ha/year), while preventing over 25 million pounds of active ingredient from being used in the environment (Ellsworth et al., 2017). While the uptake of Bt cotton and other selective technologies was critical to enabling greater reliance on natural controls like conservation biological control, the key to success was ongoing, progressive development of soft technologies that built-out the IPM strategy and the continued investments in engaged outreach and grower education to support proper integration and compatibility of practices. As such, GE crops are a powerful, selective, and therefore enabling tactical elements of IPM that, when properly integrated and

stewarded, can help maximize benefits to stakeholder while minimizing downside risks.

As already noted, structured refuges usually of the same host plant are critical components to the durability of Bt traits in GE plant systems. However, functional refuges can be supplied through novel means, the deployment of sterile insect technique (SIT) and/or pheromone-based mating disruption. Arizona cotton grower organizations in partnership with industry, university research and extension, and state and federal regulatory agencies embarked on an eradication program that permitted growers to plant up to 100% of their cotton to Bt cultivars without planted refuges starting in 2006. Refuges were supplied by targeted and proportional releases of sterile male pink bollworm moths over Bt and non-Bt fields throughout Arizona and mating disruption (Naranjo and Ellsworth, 2010; Tabashnik et al., 2010, 2012). Supported by cultural and other measures, this eradication campaign extended throughout all infested states of the U.S. and northern Mexico, resulting in the rare achievement of eradication of the pink bollworm and recent lifting of related cotton quarantines of U.S. cotton in October of 2018 (USDA, 2018).

Enabled and strengthened by the proper integration of hard technologies like GE crops, the Arizona cotton IPM strategy entailed the development, integration, and extension of no fewer than 15 other tactical building blocks (see Figure 1 in Ellsworth and Martinez-Carrillo, 2001), many rooted in knowledge-based, soft technologies (e.g., sampling plans, action thresholds, resistance management). The central, foundational tactic of conservation biological control enabled by selective technological inputs is responsible for at least 42% of the economic gains made by Arizona cotton growers (Ellsworth et al., 2017). Much of the balance of these gains (58%) are due to the hard technologies *per se*, including Bt cotton inclusive of their actual grower costs. This remarkable stability and durability of this IPM system likely emboldened growers to mount the eradication campaign and contributed in large measure to this successful outcome. Refuges, structured between 1996 and 2005 and in the form of SIT starting in 2006, and resistance management goals have also benefited by the remarkable gains in conservation biological control. Furthermore, biological control was potentially important to supporting the extirpation of the pink bollworm, the primary target pest species of Bt cotton in Arizona.

IPM OF BT EGGPLANT IN BANGLADESH AND THE PHILIPPINES

While the advent of GE crops was a transformative success story in agriculture for maize, cotton and soybean, the use of Bt crops has almost entirely been limited to these large acreage commodity crops (Shelton et al., 2017). Research and development of GE technology for "minor" crops have not been as prominent. This is unfortunate because this group of crops includes fruits and vegetables, both of which are needed for a balanced, nutritious diet and for diversified farm income. Furthermore, fruits and vegetables tend to be heavily treated with insecticides because of their diverse insect complexes, high market value, and strict cosmetic requirements (Shelton et al., 2008), resulting in what is often referred to as the produce paradox (Palumbo and Castle, 2009). The role of GE crops in an IPM strategy for many minor crops remains largely untapped but the example of eggplant demonstrates the potential benefits.

Eggplant, *Solanum melongena* L. (also known as brinjal in India and Bangladesh, and talong in the Philippines) is one of the most important, inexpensive and popular vegetable crops grown and consumed in Asia. The biggest constraint to eggplant production throughout Asia is the chronic and widespread infestation by the eggplant fruit and shoot borer (EFSB), *Leucinodes orbonalis* Guenée (Lep.: Crambidae) (**Figures 1A,B**). Infestation levels may exceed 90% and the yield loss has been estimated up to 86% in Bangladesh (Ali et al., 1980). It has been reported that 98% of Bangladeshi farmers relied solely on insecticide applications to control EFSB (Karim, 2004) and farmers spray insecticide nearly every day or every alternate day with as many as 84 applications during a 6–7 month cropping season (BARI, 1994). Such heavy reliance on insecticides, including broad-spectrum organophosphate, carbamate and pyrethroid insecticides, has been implicated in negative effects on human health and the environment (Dasgupta et al., 2005). Similarly, in the Philippines, damage by EFSB can result in yield loss of 80% and control relies primarily on frequent applications of insecticides (Francisco, 2009). Unfortunately, in resource poor areas in Bangladesh and the Philippines, these pesticides are often applied without the appropriate protective equipment, resulting in high and prolonged exposures to farmers (**Figure 1C**). Due to the high potential for pest damage, current lack of alternative tools or strategies for managing this pest, and high economic value of this crop, there is a great opportunity for leveraging GE technology as a tool for an IPM strategy. Furthermore, because EFSB is a close relative of the European corn borer which was so successfully controlled by Bt maize, it was suggested that Bt eggplant might also be an appropriate management strategy for EFSB.

The development of Bt eggplant began in 2000 by the India-based Maharashtra Hybrid Seed Company (Mahyco) under a partnership with Monsanto Company, using a *cry1Ac* gene that had already been widely used in Bt cotton in India. The *cry1Ac* gene expresses the Cry1Ac protein, which confers protection against specific lepidopteran pests, including EFSB. Research and development of the Bt eggplant included efficacy trials, and control of EFSB was demonstrated in contained greenhouse trials (Choudhary and Gaur, 2008). A partnership was developed with Mahyco, Cornell University, the United States Agency for International Development (USAID) and public sector partners in India, Bangladesh and the Philippines under the Agricultural Biotechnology Support Program II (ABSPII) in 2003. Bangladesh was the first country to approve cultivation of Bt brinjal and, on 22 January 2014, Bt seedlings were distributed among 20 farmers in four districts in Bangladesh. Due to the clear benefits of Bt brinjal for EFSB control, adoption of the GE technology has increased each year. In 2017, more than 6,000 small-scale, resource-poor farmers in Bangladesh grew Bt brinjal on their farms. In 2018, adoption increased to more than 27,000 farmers

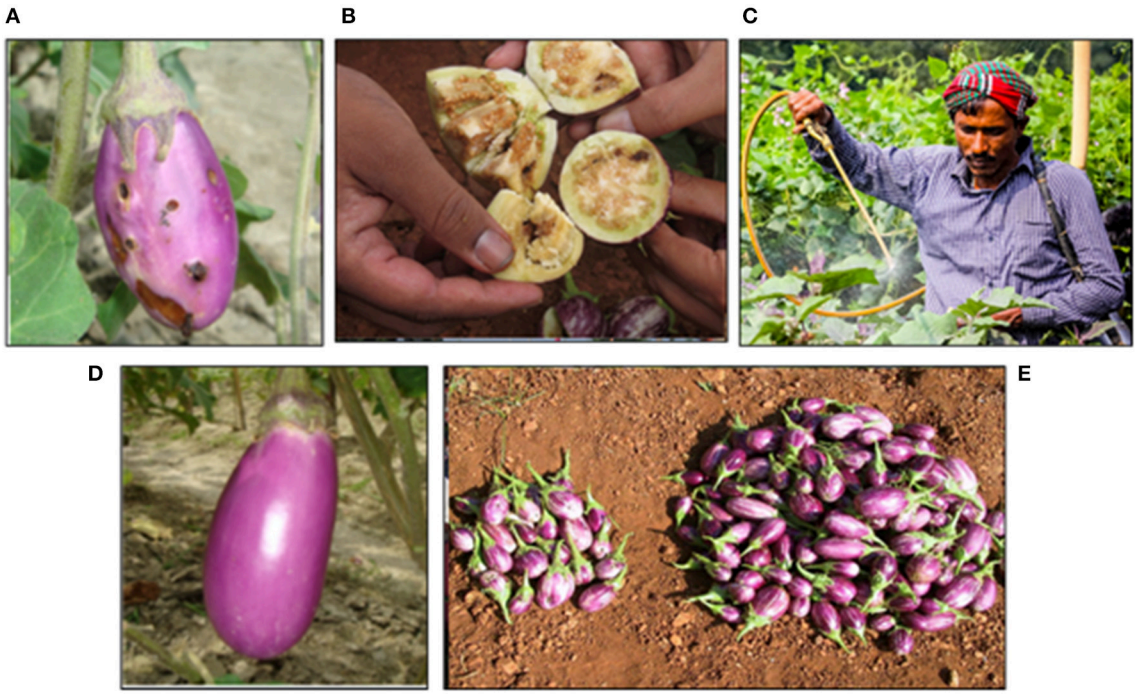

FIGURE 1 | (A) Eggplant, *Solanum melongena* L. (also known as brinjal) damaged by the eggplant fruit and shoot borer (EFSB), *Leucinodes orbonalis* Guenée (Lep.: Crambidae). **(B)** EFSB burrowing in the fruit of an eggplant. **(C)** Farmer Shahjahan spraying pesticide without appropriate personal protective equipment (i.e., gloves, mask, eye protection, etc.) in his brinjal field. **(D)** Bt brinjal line, Uttara, grown as part of a field trial in Bangladesh to demonstrate efficacy. **(E)** Bt brinjal (right) compared to non-Bt brinjal (left) as an example to demonstrate potential increased yield.

(Shelton et al., 2018). In fact, this estimate may even be higher because the distributed seed is open-pollinated and growers can save seed from the previous year.

Studies have shown that Bt brinjal provides nearly complete control of EFSB and dramatically reduces insecticide use, providing tremendous economic, health, and environmental benefits to farmers (Shelton et al., 2018) (**Figures 1D,E**). Preliminary socioeconomic studies indicate that Bt brinjal farmers have a six-fold increase in income, compared to non-Bt brinjal farmers. As with any effective host plant resistance technology for insects, the reduced need to spray for the key pest (EFSB) will have cascading effects in the agro-ecosystem and affect IPM tactics. For example, other tactics will be needed to control the complex of "sucking insect pests," but this can be done through use of more selective insecticides or through enhanced biological control through conservation of natural enemies. As shown with other cropping systems (see examples of Bt maize in Brazil and cotton in Arizona and Mexico), use of Bt plants has allowed natural enemies to play a more prominent role for control of primary and secondary pests, such as sucking insects. Studies in the Philippines have already shown important natural enemies are conserved when using Bt eggplant (Navasero et al., 2016) and many studies have shown that conservation of natural enemies through the use of Bt plants can help them control secondary pests (e.g., Tian et al., 2015). Furthermore, studies have also shown that natural enemies can contribute to delaying the evolution of Bt resistance in the key pest species (Liu et al., 2014), a win-win situation for farmers.

In Bangladesh, the Minister of Agriculture has been an outspoken and strong supporter of biotechnology and this has been an essential factor in its adoption (Shelton et al., 2017). Meanwhile, the USAID partnership program is trying to move forward in the Philippines by helping them develop and submit a strong regulatory dossier. However, in India, where research on Bt eggplant first originated and where the Genetic Engineering Committee of India approved its commercialization in 2009, Bt eggplant is still not grown because of political pressure on the Minister of the Environment and Forests resulting in a moratorium that is still in place today (Shelton, 2010).

Besides the regulatory challenges for Bt eggplant, there are other significant challenges and foremost is good stewardship. The USAID partnership program works with the Bangladesh Agricultural Research Institute (BARI) as its implementing partner. In February 2018, scientific and technical project staff conducted a 4-day workshop and training program at BARI on gene equivalency and maintaining line purity (Cornell University, 2018; Hossain and Menon, 2018). Even before the seed is delivered, it is vital that the farmer receives adequate training on this new technology. Prior to the first release of Bt brinjal, BARI conducted training and continues to emphasize that Bt brinjal needs to be treated for other insects and diseases, and non-Bt brinjal should be planted as border rows (refuge)

to delay the evolution of resistance. Monitoring for adherence to seed quality and refuge planting by farmers is critical for the sustainability of Bt brinjal. Furthermore, monitoring for changes in susceptibility of EFSB to Cry1Ac in the field is an essential component of tracking sustainability. To date, there are limited data available on baseline susceptibility, however additional studies are underway. Likewise, plans need to be developed to incorporate an additional Bt gene into lines to enhance their durability. This should be done before resistance to Cry1Ac occurs (Zhao et al., 2005) and will require regulatory adroitness and new licensing agreements with the technology provider of the dual gene event. Other strategies including pheromonal disruption are being investigated in other brinjal projects in SE Asia and, if successful and economically feasible, can be incorporated in a Bt brinjal IPM program as a complementary tactic. Meanwhile, if resistance does occur, the government will need to implement contingency plans for how to control EFSB. Unfortunately, these strategies are costlier, more labor intensive and less effective (Talekar, 2002).

Proper stewardship is a challenge in any country, but even more so in a developing country like Bangladesh that does not have experience with GE field crops and with a crop like Bt brinjal for which the farmers can save the seed. Farmer training is a vital component of the program and needs to emphasize IPM concepts to ensure the durability of this valuable product. But if the challenges associated with Bt brinjal can be overcome and sustainable solutions implemented, Bt eggplant represents a great advance in the farmer's ability to manage ESFB damage in this crop as part of an IPM approach. It also points the way forward to using biotechnology for minor crops in developing and industrial countries for control of major pests, while reducing the use of traditional pesticides.

IPM OF BT MAIZE IN BRAZIL

Maize is an important crop grown in Brazil and *S. frugiperda* (fall armyworm) is the major maize pest (Blanco et al., 2016). Pest populations have intensified over the years due, in part, to growers planting maize during a second growing season. This creates a "green bridge" that provides continuous host plants and allows *S. frugiperda* to complete up to 8–10 generations a year on maize (Storer et al., 2012). Prior to GE maize, Brazilian growers primarily controlled *S. frugiperda* with insecticides. Instead of scouting and use of economic thresholds, growers typically sprayed prophylactically every 1–2 weeks due to the polyphagous feeding habits, migratory abilities from field to field, and multiple overlapping immigrations into a field during early corn growth (Cruz, 1995). Growers have also historically increased spray rates and volumes to improve larval mortality once the larvae move to the whorl. Conversely, growers' use of aerial spray applications over larger fields has resulted in reduced spray coverage due to lower volumes delivered, which also likely decreased the effective dose against *S. frugiperda* larvae. Control practices like these in combination with the challenging biology of *S. frugiperda* has led to rapid resistance evolution to many insecticides in Brazil (Diez-Rodeiguez and Omoto, 2001).

Recent introductions of GE technology (2008–2010), targeting *S. frugiperda,* in Brazil have provided levels of crop protection in maize not previously realized by Brazilian growers. The GE maize produces various Bt proteins (including Cry1Ab, Cry1F, Cry1A.105, Cry2Ab, Vip3Aa) that are toxic (at varying levels) to *S. frugiperda* larvae upon ingestion of plant tissue. Although the high risk of resistance evolution to Bt was recognized at the time of commercialization and IRM recommendations were provided by industry, resistance has quickly evolved to multiple Bt proteins (particularly Cry1A and Cry1F) within 3–4 years (Farias et al., 2014; Omoto et al., 2016).

Rapid resistance evolution to Bt proteins is thought to be a result of the deployment of these products without meeting key assumptions for the high-dose/refuge resistance management strategy. Three assumptions should be met for this strategy: (1) recessive inheritance of resistance in pest species; (2) low initial resistance allele frequency; and (3) abundant refuges of non-Bt host plants near Bt crops promoting random mating (Tabashnik et al., 2013). GE crops deployed in Brazil to date have all violated at least one of these important prerequisites (Tabashnik et al., 2013). Low refuge compliance in Brazil is one common issue faced by all GE Bt products and, as discussed earlier, Brazil is one country where IRM is not required through regulation. Minimal industry and grower adoption of refuges contributed to the accelerated resistance evolution observed with *S. frugiperda.* Resistance allele frequency against Cry1A and Cry1F proteins also appears to have been relatively high with *S. frugiperda* populations leading to quick evolution of resistance (Farias et al., 2016; Omoto et al., 2016). Finally, proteins like the Cry1As and Cry1F are known not to be high-dose against *S. frugiperda* (Vélez et al., 2016).

One proposed resistance management solution to these Bt resistance problems has been the introduction of Bt pyramids to affected geographies like Brazil. GE pyramid products express at least two proteins that are effective against the same target insect. Due to cross-resistance among similar Bt proteins, the effectiveness of the pyramid strategy in Brazil as a resistance management tool has been limited thus far (Bernardi et al., 2015). Cross-crop resistance is another concern in diverse crop landscapes where multiple crops share similar Bt proteins. Research results suggest that if cross-crop resistance occurs among different Bt crops, landscapes like Brazil where corn, cotton, and soybean share similar Bt proteins, the selection period for cross-crop insects will be extended and thus accelerate resistance evolution (Yang et al., 2016). Therefore, rapid resistance evolution with pests like *S. frugiperda,* is likely linked to multiple factors described in this case study.

Resistance management has a limited likelihood of success if GE products like those described above are not placed into a well-understood IPM framework capable of sustaining the value of these technologies. The potential utility and contribution of IPM tactics including cultural and biological controls need to be better understood. The industry has developed several initiatives to drive the implementation of refuges and best management practices (BMPs) with growers. Industry alignment meetings led by the Insecticide Resistance Action Committee (IRAC) were initiated in 2015 to develop BMPs for maize, soybean and cotton

farmers. Industry also developed several pilot programs with growers to educate and provide incentives for adopting refuge, though these have resulted in minimal uptake to this point. Although research continues to refine management tactics to use with GE and non-GE refuge crops, tropical geographies like Brazil that harbor pests like *S. frugiperda* will challenge IPM and IRM strategies. Socioeconomic factors should be combined with agricultural systems knowledge to develop an industry framework that drives adoption of key IPM and IRM practices. In addition, regulation that requires critical resistance management tactics like the planting of refuges should be pursued. Until either or both of these approaches are further developed, deploying new GE technologies in countries like Brazil should proceed with caution.

IPM OF BEAN GOLDEN MOSAIC VIRUS IN COMMON BEAN IN BRAZIL

Common bean (*Phaseolus vulgaris* L.) is an important staple food in Brazil and other countries in Latin America. Similar to brinjal, common bean is an orphan crop that can utilize GE technology to complement the IPM approach for managing bean golden mosaic virus (BGMV). BGMV is the causal agent of the most destructive viral disease of common beans in Brazil. It is efficiently vectored by the whitefly, *B. tabaci*, which is also a significant insect pest for this and several other crops, especially in tropical areas. BGMV causes stunted growth, yellowing and flower abortion, and high yield losses (Anderson et al., 2016). Traditional pest control tactics for the insect vector are limited to chemical pesticide application, and overuse of pesticides on common beans is a common problem leading to environmental effects and insect resistance problems (Bonfim et al., 2007).

GE common bean was modified using RNAi technology to develop a BGMV resistant variety by Brazilian Agricultural Research Corporation (Embrapa) (Bonfim et al., 2007; de Faria et al., 2016). BGMV resistant common bean was granted commercial approval by Brazil in 2011 [Comissão Técnica Nacional de Biossegurança (CTNBio), 2011] and was registered and protected as cultivar BRS FC401 RMD by the Brazilian Ministry of Agriculture, Livestock and Food Supply in 2016 (Souza et al., 2018). GE common bean offers an opportunity to farmers to control this viral pathogen without chemicals. However, there remain several key challenges for successful integration of this technology into a sustainable IPM plan. Following regulatory approval, the current challenge is to successfully insert this GE trait into commercial varieties that are optimized for the different regions (Souza et al., 2018). Additionally, IPM and farm management practices are being optimized and farmer training is being offered to ensure sustainable use and durability of the trait. For example, management strategies including implementing a whitefly host-free period (elimination of hosts for both virus and whitefly), designating sentinel areas (where common bean fields are planted early in the season to screen for the presence and abundance of viruliferous whitefly populations), and optimizing planting time and chemical control practices are all valuable components of the emerging IPM plan. These tactics are important to reduce damage by whitefly due to direct feeding as well as deposition of honeydew on which mold fungi can grow and reduce photosynthesis. Additionally, it is important to reduce areawide pressure of whitefly as a disease vector because, while BGMV is the most devastating virus, it is not the only whitefly transmitted virus to common beans (Brown et al., 2015). New geminiviruses [*Macroptilium* yellow spot virus—MaYSV; Soybean chlorotic spot virus—SoCSV; and *Macroptilium* yellow vein virus—MaYVV (Sobrinho et al., 2014)] are a threat to common beans in Northeastern Brazil, and the flexivirus, Cowpea mild mottle virus, is a destructive disease of common beans (de Faria et al., 2016).

Building professional capacity through farmer training, and developing an alert system to quickly identify if a threshold for pest population or viral pathogen load is being exceeded will also be critical to success. Because the GE common bean varieties have not yet been commercialized, this work to optimize management practices and increase farmer training is being conducted with growers on small plots (up to a half hectare). There have been encouraging results with implementing whitefly host-free periods and using an alert system to evaluate the real need for chemical control. Going forward, the use of whitefly monitoring/reporting system and the sentinel areas will help growers to make the correct decision about whether to grow common beans or switch to an alternative crop to maximize income with lower risks of crop losses. GE common bean with resistance to BGMV will help to diversify the tool box for IPM in Brazil, and an integrated approach to pest management of whitefly is essential for achieving agricultural and environmental sustainability, food security and grower profitability. IPM practices (including whitefly monitoring, sentinel areas, pest free periods, etc.) must be continued and leveraged to enable decision-making and successful integration of a sustainable IPM plan.

INTEGRATED WEED MANAGEMENT (IWM) WITH HERBICIDE TOLERANT CROPS

Weed management strategies have not changed greatly in the last five decades. Despite the adoption of GE crops with HT traits, weed management arguably is still largely, if not exclusively, based on herbicides. HT crops have many advantages, and the benefits of being able to use herbicides that would cause unacceptable phytotoxicity to a crop (e.g., glyphosate) are clear. However, to date, HT traits are largely limited to conferring tolerance to a few herbicidal active ingredients, and a small subset of commercial commodity crops. Therefore, many opportunities to expand the portfolio of HT traits in crops with this technology remain, considering that the availability of herbicides for use in high value crops such as fresh vegetables is limited. If HT traits were available in some high value crops, the effectiveness of weed control would improve greatly, the costs of weed control would decline and the quality of the crop would increase (Gianessi, 2013).

Despite the unprecedented success of HT technology for weed management, successful implementation and sustainability of this technology presents many challenges, including the evolution of herbicide resistance in key weed species. Success of HT crops is seen as increased simplicity of weed management, improved time management and reduced costs; farmers as a result, became increasingly unwilling to adopt integrated weed management (IWM) practices including the need for multiple herbicidal modes of action to address evolved herbicide resistances in weeds (Frisvold et al., 2009; Norsworthy et al., 2012). These challenges highlight the need for diverse, well-designed and proper IWM plans. It is important to recognize that herbicide resistance evolution is not necessarily a reflection on the cultivation of HT crops. Rather, herbicide resistance has been a prominent problem for agriculture since the beginning of herbicide use (Heap, 2019). The issue of evolved herbicide resistance in key weeds reflects the fact that herbicides have been the principle tactic for weed control for more than 45 years and the inclusion of alternate strategies for weed management has declined steadily over the same period of time (Jussaume and Ervin, 2016; Owen, 2016). For example, glyphosate has been applied on the majority of row crop acres in the US for more than two decades. While there are many reasons and justifications for this approach including improved time management and efficiency, reduced costs for weed control, as well as increased effectiveness, simplicity and convenience, the ecologically narrow focus of one approach unsurprisingly resulted in rapid and widespread evolved resistance to glyphosate within important weed species such as *Amaranthus tuberculatus* J. D. Sauer, *A. palmeri* S. Wats, and *Conyza canadensis* (L.) Cronquist (Owen et al., 2015). This clearly demonstrates why weed management in row crops is not sustainable if based primarily on a single herbicide.

Because herbicides will likely continue to play a significant role in weed management in the future, designing robust management plans for weeds will be important for sustainability of HT crops. Unfortunately, strategies associated with IPM for insect pests are often not applicable for weeds (Owen, 2013, 2016). For example, concepts such as action thresholds for insect damage have no utility in weed management, given the growth plasticity of weeds, the high amount of seeds produced, and the long life of seeds in the soil seedbank. In fact, often the decision to allow weeds to remain uncontrolled because the population density is below a theoretic economic injury level at one point in time will result in greater weed problems in the future. Similarly, IPM programs for insects and diseases are typically developed around one pest species; whereas for weed communities found in crop fields, many species, each with different ecological characteristics and management requirements need to be considered. For example, different weed species affect the crop at different times of the growing season which complicates the timing of control tactics. Furthermore, many weeds have numerous germination events, each of which requires control while insect pests tend to have fewer emergence events that simplifies the timing of control tactics. Finally, with weeds, the pest targets are closer morphologically, phenologically, physiologically and biologically to crops than insect or diseases, which presents additional challenges and limits the flexibility of control tactics. Nevertheless, the need for sustainable and durable tactics for weed control is important. The development of an IWM strategy, which includes diverse tactics other than herbicides for weed control, complements the concept and foundational approach of IPM programs developed for other pest complexes (Swanton and Weise, 1991; Swanton et al., 2008; Owen, 2016).

Diverse IWM strategies include, but are not limited to, cultural and biological tactics that can supplement mechanical and herbicide-based weed management approaches and will be important components of successful weed management programs in the future (Meissle, 2016; Owen, 2016). Examples of diverse strategies that supplement an herbicide-based weed management plan include, but are not limited to harvest weed seed destruction (Walsh et al., 2018) and more diverse crop rotations employed in a crop system (Blackshaw et al., 2008). Related to seed destruction for example, Walsh et al. (2018) illustrated the successes of reducing the weed seedbank, and describes several tactics that can be used during crop harvest that destroy weed seeds thus improving weed management efforts in the future. Similarly, Blackshaw et al. (2008) demonstrated the positive effects of using diverse crop rotations [in this case, GE canola (*Brassica napus* L.) and forages such as alfalfa (*Medicago sativa* L.)] for reducing weed population densities and improving overall weed management in a cereal-based crop production system. While it may be simpler to depend on a few weed management practices, the key to sustainability will be for all entities involved in weed management, private, commercial and government, to consider more diverse weed management approaches. For example, Iowa, a key US state for maize and soybean production, currently is developing a state Pest Resistance Management Plan established by an inclusive committee that represented all agricultural groups and sponsored by the Iowa Department of Agriculture and Land Stewardship and the Iowa State University College of Agriculture and Life Sciences (Iowa Pest Resistance Management Program, 2018). The plan provides guidelines for establishing management programs for herbicide-resistant weeds and consists of pilot projects demonstrating community-based weed management. However, the specifics of the conceptualized diverse and community-based management plans for herbicide-resistant weeds have yet to be developed and implemented.

Herbicide-resistant weeds represent a "wicked" problem, in that there is no single strategy for weed management and new technological advances alone will not resolve the issue (Ervin and Jussaume, 2014; Ervin and Frisvold, 2016). Herbicide resistant weeds are very mobile within an agricultural community, and while local solutions should be adaptable to an individual grower's needs, they must align with the broader weed management goals at a landscape or regional level (Ervin and Jussaume, 2014). Confounding the effort to manage those weeds are multiple herbicide resistances in a majority of key weed populations (Owen et al., 2015). While some farmers may recognize the importance of community involvement with

BOX 1 | Challenges and solutions for successful implementation of an integrated pest management (IPM) approach.

Challenges	Solutions
IPM is knowledge-driven. Information about the biology and ecology of pests and natural enemies, development and testing of appropriate tools and strategies, and training of farmers to use these tools appropriately is needed for successful deployment.	A strong partnership is needed between industry, regulators and farmers, with an emphasis on farmer training. Training should emphasize IPM concepts, benefits and limitations of tools and pest management strategies, and the importance of compliance. Innovative solutions and best management practices aimed at sustainability must continue to be developed, adapted and implemented on a local scale. Individual and areawide benefits and challenges need to be identified, discussed, and promoted early in the development and deployment processes of GE technologies When changes in management strategies are needed, a communication plan and intensive education program are needed to transfer knowledge.
IPM plans need to serve environmental, human health, and social goals while being economically beneficial to the producer.	Incentives are needed for the implementation of an IPM strategy that maintains the ecological infrastructure, facilitates the implementation of crop rotations, and supports the application of environmentally friendly pest control systems to gain compliance. The IPM approach in each region should consider the cultural and socio-economic contexts. Farmer training and education can put the short-term costs into context with the long-term benefits of implementing a sustainable approach. An industry and government partnership for IRM regulation ensures compliance to IRM and IPM practices.
GE technology is often perceived as a "silver bullet."	GE crops are implemented within a comprehensive IPM strategy. A range of efficient and economically feasible options including, but not limited to the GE crops, needs to be available for a given production system and region. Understanding that pest shifts may occur, integrated solutions should not only address the current pest spectrum, but should also consider the possibility of pest shifts. GE traits should complement a broader IPM strategy filled with companion and compatible selective tactics for all the other pest challenges in the system. Farmer training and education can transfer knowledge, leverage the experiences gained with GE crops, and allow for a better understanding of the benefits and limitations of each technology.
Regulatory restrictions, high costs for developers, and long approval timelines for GE crops can restrict the availability of options for farmers and greatly reduce and delay benefits to society.	A science-based approach for the safety assessment and regulation of GE crops is needed. Public discussions need to move from a focus on GE technology in general to a focus on cropping systems, their problems, and potential solutions, including GE crops. Collaboration and communication among consumers, academics, industry, and government, that leverage the experience and familiarity gained with the cultivation of GM crops over the last twenty years, is needed to transfer knowledge, and allow for a better understanding of the benefits and limitations of GE crops.

regard to herbicide resistance management, some feel that any efforts put forward will be for naught, as their neighbors will not participate in the effort (Doohan et al., 2010; Barrett et al., 2017). As previously discussed by Davis and Frisvold (2017), "The efficacy of any pesticide is an exhaustible resource that can be depleted over time. For decades, the dominant paradigm—that weed mobility is low relative to insect pests and pathogens, that there is an ample stream of new weed control technologies in the commercial pipeline, and that technology suppliers have sufficient economic incentives and market power to delay resistance supported a laissez faire approach to herbicide resistance management. Earlier market data bolstered the belief that private incentives and voluntary actions were sufficient to manage resistance. Yet, there has been a steady growth in resistant weeds, while no new commercial herbicide modes of action (MOAs) have been discovered in 30 years" (Davis and Frisvold, 2017). Unless there is a community-based effort put forth to manage herbicide resistance that goes beyond using herbicides, it is unlikely that any effort will be successful. Therefore, while GE crops may offer great opportunities for weed control in agriculture, there remains a critical need to adopt diverse tactics other than herbicides to manage resistant weeds and to reduce the risk of herbicide resistance evolution where it has not yet become a problem.

DISCUSSION

The goal of an IPM strategy is to support the sustainable production of high quality crops while minimizing environmental impacts attributable to pests or pest management practices. While the benefits of using an IPM approach are evident, as outlined in the case studies above, implementation of IPM can be very challenging for several reasons (**Box 1**) (Meissle, 2016). One common theme among the case studies presented is that a successful IPM or IWM strategy leverages a diversified approach. GE crops should not be viewed as a "silver bullet," and while their success may seem like an infallible solution to control pests in the short run, durability and sustainable use requires a long-term vision. As can be seen based on the many years of experience using Bt and HT traits, insects and weeds will inevitably evolve resistance over time. Part of the goal of the IPM plan is to diversify the approaches to pest management, and limit the dependence on one single technology. Just as it is crucial for IPM practices (including

whitefly monitoring, sentinel areas, pest free periods, etc.) to be continued for whitefly control in common beans in Brazil, it is equally critical that comparable IPM practices are developed, optimized and maintained for all crops and pests. Knowledge and understanding of the technology, pest, crop, region, alternative tools and even social contexts are critical for the success of an IPM plan, because if there is insufficient understanding of the technology and how best to integrate it into an IPM system, the durability of the technology may fail. In addition, if there is not adequate training and engagement of farmers to recognize the short- and long-term benefits of the management plan, the technology may fail due to lack of compliance. Incentives may be needed to gain producer compliance with best management and resistance management requirements, and often farmer training is needed to demonstrate the short-term and long-term benefits of implementing a sustainable approach.

Likewise, training stakeholders about how best to integrate and use GE crops in their existing agricultural system is critical. While industry tends to focus on discovery, research and development and promoting the value of GE traits, there is a huge responsibility for institutions (e.g., government, public, and private) to make the investments necessary to develop the systems that consider not only the technical solutions possible, but also the cultural and socio-economic contexts. As suggested by Stern et al., "one reason for the apparent incompatibility of biological and chemical control is our failure to recognize that the control of arthropod populations is a complex ecological problem. This leads to the error of imposing insecticides on the ecosystem, rather than fitting them into it" (Stern et al., 1959). On the other hand, if the technology and tactics are fit to the existing system, and appropriate training is provided to stakeholders, there is a much higher chance of success and sustainability over time.

Because of these (and other) challenges for successful implementation of an IPM approach, pest control based on broad spectrum chemicals is often perceived as the easier, more economic, and most efficient short term approach used for pest management in large-scale farming operations. To promote continued research, expand implementation, and highlight the value of using an IPM strategy, a joint effort among governments, label organizations, growers, grower associations, and the seed and pesticide industries is critically needed. Most of the major successes in gaining grower support for resistance management over the past 50 years were preceded by pest resistance-related economic failures and the solutions involved a strong partnership between industry, regulators and farmers. Innovative solutions and BMPs aimed at sustainability must continue to be developed in particular for crops and regions where there is high resistance risk (e.g., tropical production systems), or grower adoption of resistance management requirements has failed.

The benefits of a successful IPM strategy, including reduced application of broad spectrum chemical pesticides, more durable pest management in ecologically balanced crop production systems, and reduced risks to human health and the environment, are clear. Sustainable, eco-rational IPM strategies rely on a diversified portfolio of tactics, of which GE crops represent a valuable tool. By leveraging the experiences gained with GE crops, understanding the limitations of the technology, and considering the successes of GE traits in IPM plans for different crops and regions, we can enhance the durability and versatility of IPM plans for future crops.

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

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When Policy Meets Practice: The Dilemma for Guidance on Risk Assessment Under the Cartagena Protocol on Biosafety

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The Conference of the Parties serving as the meeting of the Parties (COP-MOP) to the Cartagena Protocol on Biosafety to the Convention on Biological Diversity decided years ago to undertake the development of guidance on risk assessment of living modified organisms (LMOs) resulting from modern biotechnology, in order to assist the Parties to the protocol to conduct risk assessments in line with the principles and methodology described therein. After many years of working through *ad hoc* technical expert groups (AHTEG) and open-ended online forum discussions, including an extensive process to test and revise the guidance document, the COP-MOP did not decide to endorse the last version of the document when it was finally presented to them. A failure to achieve consensus that the guidance, as it had evolved, is relevant and useful is seen as a potential setback for many Parties to the protocol with little to no experience with risk assessment. There are a number of reasons for the lack of success in this attempt to develop useful guidance on risk assessment, including a poorly defined and shifting purpose, misplaced expertise, and a misguided testing process, mostly perpetuated by the constraints of using processes of the Convention. These problems with the development of the Guidance on Risk Assessment of LMOs are explored here in an effort to elucidate the missteps that should be avoided and the lessons that can be learned. Most prominent is a need to rely upon the expanding past and present experiences with actual cases of risk assessments of LMOs, if there is to be any further attempt to develop guidance on risk assessment under the Convention and its protocol.

Keywords: biosafety, cartagena protocol on biosafety, risk assessment guidance, AHTEG, COP-MOP, genetically modified organisms, biotechnology regulation

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INTRODUCTION

The Cartagena Protocol on Biosafety to the Convention on Biological Diversity is an international agreement that provides a regulatory framework for the safe handling, transport, and use of '*living modified organisms (LMOs) resulting from modern biotechnology that may have adverse effects on the conservation and sustainable use of biodiversity*' (SCBD, 2000). The dual-purpose of the Cartagena Protocol on Biosafety (hereinafter referred to as "the Protocol") as described in its introduction is to create *'an enabling environment for the environmentally sound application of biotechnology, making it possible to derive maximum benefit from the potential that biotechnology has to offer, while*

minimizing the possible risks to the environment and to human health'. The Protocol entered into force in 2003 and since then has been ratified by 171 countries as Parties. Negotiations among the Parties over the implementation of the articles of the Protocol have since taken place during nine "Conference of the Parties serving as the meeting of the Parties (COP-MOP) to the Protocol, and negotiations will continue into the foreseeable future.

The Protocol has clearly significantly shaped the development of most national biotechnology regulatory frameworks, with impacts on a range of issues including environmental risk assessment (ERA), socio-economic considerations, and liability and redress, particularly in developing countries (McLean et al., 2012; Adenle et al., 2018). The dominating presence of the European Union (EU), which serves as a "Party" to the Protocol as does each of its 28 member states, with a decidedly first-world, highly precautionary stance on genetically modified organisms (Science for Environment Policy, 2017; Eriksson, 2018), has been particularly influential in these negotiations (Paarlberg, 2006; Adenle et al., 2018). The Protocol has serious implications for global agricultural trade and food security, making it critical that it is implemented in a practical way without perpetuating overly strict or unobtainable regulatory hurdles while effectively promoting the conservation and sustainable use of biodiversity.

One of the most significant discussions taking place over the years of negotiation concerns risk assessment, covered in Articles 15 and 16 of the Protocol. Annex III of the Protocol outlines the objective, use, general principles, methodology, and points to consider for risk assessment (SCBD, 2000). While most Parties agree that the general principles and methodology provided in Annex III (see **Box 1**) represent what is typically followed for risk assessments of LMOs, some Parties saw a need to develop further guidance on "specific aspects" of risk assessment. At COP-MOP4 in 2008, the Parties agreed to establish an open-ended online forum and *ad hoc* Technical Expert Group (AHTEG) on Risk Assessment and Risk Management to develop such guidance (Decision BSIV/11)[1] Eight years later, after various drafts of the Guidance were presented and not endorsed by the Parties over three more COP-MOPs, and frequently polarized online forum discussions and face-to-face meetings of the AHTEG, and a lengthy testing and revision process (see **Figure 1**), the latest draft of the Guidance on Risk Assessment of LMOs (hereinafter referred to as "the Guidance") was completed, published and presented at COP-MOP8 in December 2016. There, the Parties decided to "take note of," but did not "endorse" (nor "welcome," nor "acknowledge") the Guidance, and the AHTEG on Risk Assessment and Risk Management (hereinafter referred to as "the AHTEG"), having completed its mandate, came to a close.

The final version of the Guidance presented at COP-MOP8 includes six sections divided into three parts (**Box 2**), beginning with a roadmap for risk assessment (hereinafter referred to as "the Roadmap"), which is Part I. The Roadmap is meant to "elaborate on how to undertake a risk assessment" and it is the core of the Guidance (Gaugitsch, 2016). It has been the main topic of discussion during the AHTEG meetings, in the online forums, in the testing process, and at the COP-MOPs. Part II is additional guidance on specific types of LMOs (mosquitos; trees) and traits (stacked genes; abiotic stress resistance); and Part III is additional guidance on monitoring of LMOs after release into the environment. The last version

Box 1 | Annex III of the Cartagena Protocol on Biosafety, Paragraphs 3-7, 8a-f.

Risk assessment general principles and methodology

General principles

3. Risk assessment should be carried out in a scientifically sound and transparent manner, and can take into account expert advice of, and guidelines developed by, relevant international organizations.

4. Lack of scientific knowledge or scientific consensus should not necessarily be interpreted as indicating a particular level of risk, an absence of risk, or an acceptable risk.

5. Risks associated with living modified organisms or products thereof, namely, processed materials that are of living modified organism origin, containing detectable novel combinations of replicable genetic material obtained through the use of modern biotechnology, should be considered in the context of the risks posed by the non-modified recipients or parental organisms in the likely potential receiving environment.

6. Risk assessment should be carried out on a case-by-case basis. The required information may vary in nature and level of detail from case to case, depending on the living modified organism concerned, its intended use and the likely potential receiving environment.

Methodology

7. The process of risk assessment may on the one hand give rise to a need for further information about specific subjects, which may be identified and requested during the assessment process, while on the other hand information on other subjects may not be relevant in some instances.

8. To fulfill its objective, risk assessment entails, as appropriate, the following steps:

(a) An identification of any novel genotypic and phenotypic characteristics associated with the living modified organism that may have adverse effects on biological diversity in the likely potential receiving environment, taking also into account risks to human health;

(b) An evaluation of the likelihood of these adverse effects being realized, taking into account the level and kind of exposure of the likely potential receiving environment to the living modified organism;

(c) An evaluation of the consequences should these adverse effects be realized;

(d) An estimation of the overall risk posed by the living modified organism based on the evaluation of the likelihood and consequences of the identified adverse effects being realized;

(e) A recommendation as to whether or not the risks are acceptable or manageable, including, where necessary, identification of strategies to manage these risks; and

(f) Where there is uncertainty regarding the level of risk, it may be addressed by requesting further information on the specific issues of concern or by implementing appropriate risk management strategies and/or monitoring the living modified organism in the receiving environment.

[1] All of the COP-MOP meeting documents referenced throughout this manuscript and other relevant information can be found online through the Biosafety Clearing House of the Cartagena Protocol on Biosafety. https://bch.cbd.int/protocol/.

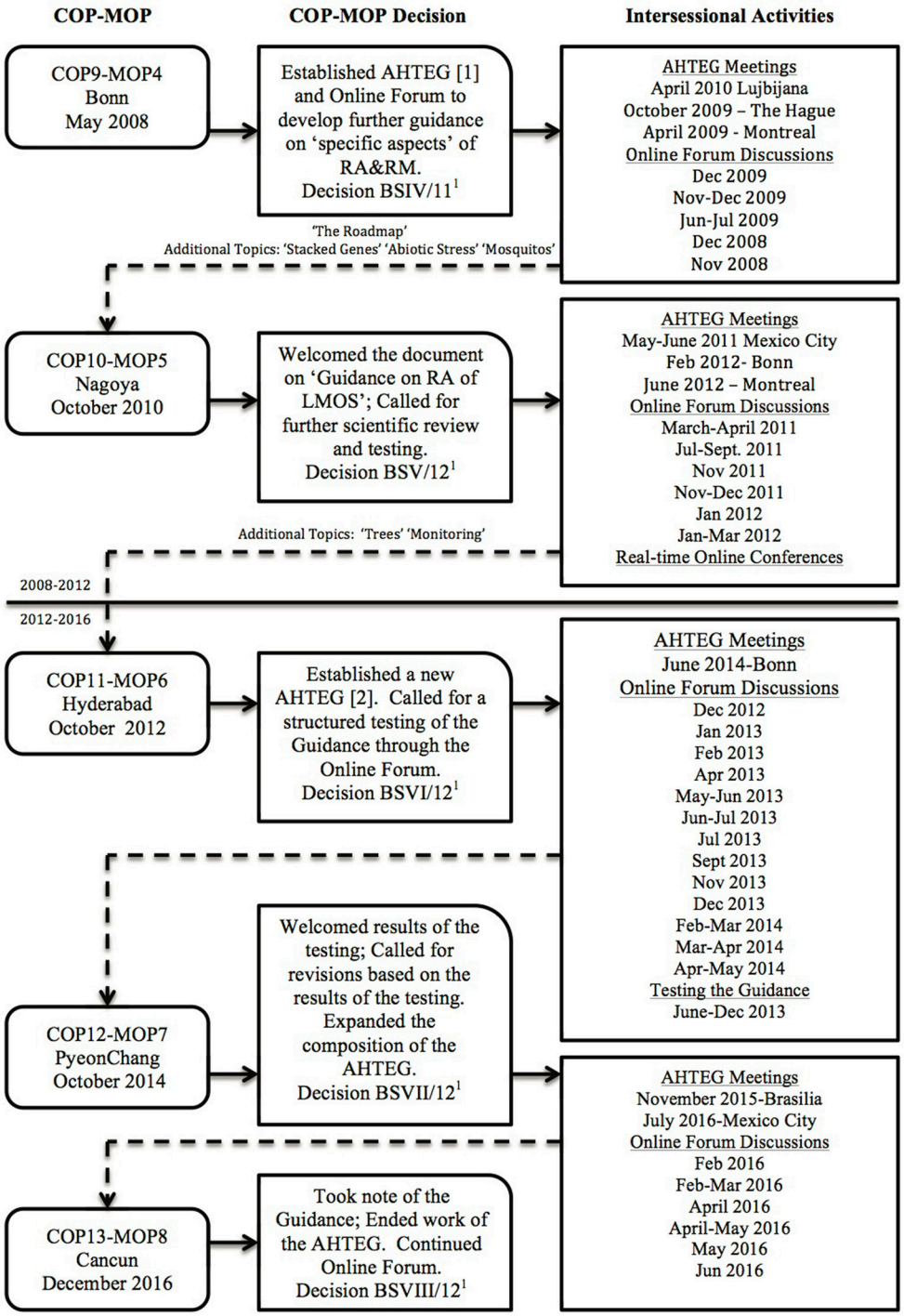

FIGURE 1 | A schematic showing the timeline and activities for the development of the "Guidance on Risk Assessment of LMOs" under the Cartagena Protocol on Biosafety.

of the Guidance as it was presented for discussion at COP-MOP8 can be found as an annex to the COP-MOP8 official meeting document UNEP/CBD/BS/COP-MOP/8/8/Add.1[1], and the same document is available as Issue 4 in the Biosafety Technical Series of the Biosafety Clearing House[1]. To satisfy the concerns of some Parties, a disclaimer can also be found there:

"*Note: This publication is the outcome of the ad hoc Technical Expert Group on Risk Assessment and Risk Management at its*

Box 2 | The guidance on risk assessment of living modified organisms.

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Part II. Specific types of LMOs
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5. Risk Assessment of Living Modified Mosquitoes Species that Act as Vectors of Human and Animal Diseases
Part III 6. Monitoring of Living Modified Organisms Released Into the Environment

meeting in July 2016. The views reported in this publication were not considered, discussed or otherwise approved or adopted by the Conference of the Parties serving as the meeting of the Parties to the Cartagena Protocol on Biosafety and do not necessarily represent the views of the Parties to the Cartagena Protocol on Biosafety."

The document, although it was not endorsed, remains a "voluntary" guidance available for use by any Party and others who would choose to use it, although it is in no way recommended or required for a Party to follow this guidance.

In the discussions leading up to and during COP-MOP8, some Parties took the position that the Guidance was useful and practical and should be endorsed, while Parties unwilling to endorse it were critical of the process by which the document was developed, particularly because it did not allow ample opportunity for Parties to review the latest version of the Guidance before deciding to endorse or not at COP-MOP8. There were also serious concerns that the contents of the document go beyond what is consistent with Annex III of the Protocol and do not represent the years of experience gained by the Parties who have conducted actual risk assessments on LMOs. Because of this, the overall usefulness of the Guidance for the implementation of the Protocol was in question. Those Parties seemed to share an opinion that, in spite of the claims that the Guidance was not prescriptive and does not impose any obligations upon the Parties, endorsement by the COP-MOP would imply that the Guidance was recommended, if not required, for use by Parties. Because the Guidance would be more difficult to implement than more practical existing approaches to risk assessment being followed in some countries, this would likely hinder rather than enable effective risk assessment for decision-making, especially in countries with little or no experience.

This outcome was a disappointment to the Parties that called for and still perceive a need for detailed guidance on risk assessment, particularly those with little to no experience assessing the risks of LMOs currently. It is both disappointing and perplexing to all Parties to see so much effort, energy, time, and money invested into a process that failed to result in an acceptable outcome. How could this have happened? Herein are a number of observations about the process and outcome that might explain the fate of the Guidance on Risk Assessment of LMOs, and some lessons learned to help shape the process should there be attempts to develop similar guidance in the future.

A QUESTION OF PURPOSE FOR THE GUIDANCE

The intent and purpose for the Guidance, and in particular the Roadmap, seemed to evolve significantly over the years. The topic of guidance on risk assessment was taken up early at COP-MOP2 where it was decided to establish an initial AHTEG that then met in Rome in November 2005. The Terms of Reference of the Rome AHTEG as described in Decision BSII/9[1] included to:

'*consider the nature and scope of existing approaches to risk assessment based on national experiences and existing guidance materials;* and '*evaluate the relevance of existing approaches and guidance materials to risk assessment under the Protocol and identify gaps in those existing approaches and guidance materials.*

In the decision from COP-MOP2 (Decision BSII/9[1]), the COP-MOP acknowledged that:

…'any guidance on risk assessment and risk management developed by the Conference of the Parties serving as the meeting of the Parties to the Protocol should support a harmonized approach, in accordance with Annex III of the Protocol, taking into account internationally agreed principles and techniques developed by relevant international organizations and bodies.'

The Rome AHTEG concluded that developing general guidance was not a priority given the amount of material already available, which needed to be collected, organized and made available (UNEP/CBD/BS/COP-MOP/3/INF/1[1]). In Decision BSIII/11[1], after the report from the Rome AHTEG, the Parties called on the Secretariat to collect existing information on risk assessment and make it available. The importance of this request, however, was lost when it became subsumed in the work that ensued on the development of guidance on risk assessment in the coming years.

A decision was later made at COP-MOP4 to form another AHTEG and an online forum and to begin work to develop guidance. The original terms of reference for the AHTEG was laid out in the annex to the COP-MOP4 decision on risk assessment (Decision BSIV/11[1]):

'Develop a "roadmap", such as a flowchart, on the necessary steps to conduct a risk assessment in accordance with Annex III to the Protocol and, for each of these steps, provide examples of relevant guidance documents;'

'Prioritize the need for further guidance on specific aspects of risk assessment and define which such aspects should be addressed first, taking also into account the need for and relevance of such guidance, and availability of scientific information;'

'Define an action plan to produce modalities for development of the guidance documents on the specific aspects that were identified as priorities.'

The "prioritization of topics" for further guidance, i.e., "specific aspects" of risk assessment in addition to the Roadmap, was part of the AHTEG's original mandate. The earlier, 2005 Rome AHTEG had also acknowledged that there may be a need for specific guidance in the future, and in discussing specific gaps in existing approaches and guidance materials, the Rome AHTEG listed a wide range of examples of specific areas where "existing guidance may not be sufficient" (UNEP/CBD/BS/COP-MOP/3/INF/1[1]). The list described what were then new techniques, product concepts, and new or less familiar issues to regulators and risk assessors. Over time, a similar reasoning was applied in proposing more new topics for guidance. The AHTEG, at its first meeting after COP-MOP4, engaged in a "priority setting exercise" which resulted in a list of 14 "prioirtized topics for the development of guidance" (Annex II of UNEP/CBD/BS/COP-MOP/5/INF/13[1]). The methods to rank the topics was not described in the report from the AHTEG meeting, and it seemed to be based primarily on the number of requests for guidance on a certain topic by some Parties. The AHTEG then defined *"an action plan to produce modalities for development of the guidance documents"* on the topics, which apparently was to work in subgroups and actually draft guidance on the top priority topics, in parallel with the Roadmap, with input from the online forum.

First drafts of the Roadmap as well as the additional guidance on stacked genes, abiotic stress and mosquitos were developed in the interim between COP-MOP4 and COP-MOP5 (UNEP/CBD/BS/COP-MOP/5/INF/13[1]; UNEP/CBD/BS/COP-MOP/5/INF/15[1]), and additional guidance on trees and monitoring was added in the interim between COP-MOP5 and COP-MOP6 (UNEP/CBD/BS/COP-MOP/6/INF/10[1]) (see **Figure 1**). It was also between COP-MOP5 and COP-MOP6 that the AHTEG decided to organize the guidance into three parts, placing the most recently developed additional guidance on Monitoring into a "Part" separate from the other additional guidance. Before COP-MOP8, the AHTEG prioritized two additional topics for guidance: "living modified fish" and "synthetic biology," and developed outlines for guidance on these topics. These outlines were presented in the report to COP-MOP8 (UNEP/CBD/BS/COP-MOP/8/8/Add.1[1]) with the last version of the Guidance, where there was no decision to pursue these two additional topics.

Regarding the Roadmap section of the Guidance specifically, in the analysis from the open-ended online forum discussions presented to COP-MOP5 (BS/COP-MOP/5/INF/12[1]), after the first 2 years of work on the Guidance, the intent of the Roadmap specifically was further elaborated as follows:

'… the roadmap should be a practical guide to assist risk assessors and decision makers on how to implement the provisions set out in the Annex III of the Protocol.'

'The roadmap is envisaged to provide additional detailed guidance on how to conduct risk assessment of LMOs …'

'Furthermore, the roadmap is to serve as a reference to guidance materials that are relevant to each step or point to consider.'

After the discussions that took place at COP-MOP5, in the COP-MOP5 decision (Decision BSV/12[1]) the purpose of the Guidance appeared to shift noticeably from a "practical" and "detailed" guidance on risk assessment to a reference document:

"… its objective is to provide a reference that may assist Parties and other Governments in implementing the provisions of the Protocol with regards to risk assessment, in particular its Annex III …"

In the latest version of the Guidance (BS/COP-MOP/8/8/Add.1[1]), there is a further attempt to emphasize the more general applicability of the Roadmap by the addition of text in the "background" section of the Roadmap itself:

'The Roadmap introduces basic concepts of risk assessment rather than providing detailed guidance for individual case-specific risk assessments. In particular, the "elements for consideration" listed in the Roadmap may need to be complemented by further information during an actual risk assessment.'

The Guidance in its current form may "provide a reference for risk assessment" of LMOs as stated in the COP-MOP5 decision, and "introduce basic concepts" as stated in the background to the Roadmap in the most recent version; yet it may not be useful as a harmonized "roadmap" or "guide" to assist risk assessors, as seemed to be its original intent. Rather than guidance based on an agreement about what is actually done in risk assessments based on experience, the Guidance attempted to represent many, varying opinions expressed by the participants of the AHTEG and the online forum about what "should be done" in risk assessment. In the note by the Executive Secretary on Risk Assessment and Risk Management for COP-MOP8 (UNEP/CBD/BS/COP-MOP/8/8[1]), it states: "*The AHTEG endeavored to reconcile the different views coming from the Online Forum by following an inclusive approach to explore all possibilities to reach a middle ground on the outstanding issues*." In fact, the resulting Guidance is a compromise document that attempts to merge some irreconcilable points of view, including views on many non-technical issues, without maintaining a connection to the source of those different views. The reports from the AHTEG meetings frequently indicate where the AHTEG had "agreed," when in fact there was compromise necessitated by the need to keep the process moving, and secured without consensus among experts.

MISPLACED EXPERTISE TO DEVELOP THE GUIDANCE

Party Members of the AHTEG

In order to understand the challenge for reaching a meaningful agreement in developing the Guidance, it is useful to consider the history of the AHTEG, its composition and membership. There were two phases of the AHTEG (actually two separate AHTEGs with some overlap of individuals as members) that

worked on the Guidance, from 2008 to 2012 and 2012 to 2016 (see **Figure 1** and **Table 1**; a list of all AHTEG members can be found on the Biosafety Clearing House[1]). The "Party members" of the AHTEG were individual experts nominated by their Parties and selected by the Executive Secretary, more-or-less in accordance with the consolidated modus operandi of the Subsidiary Body on Scientific, Technical, and Technological Advice (SBSTTA) (see **Box 3** and **Box 4**). Therefore, the composition of the AHTEG as selected from the list of nominated experts attempted to consist of equal representation from each of the five regional groups of the United Nations "*with due regard to geographical representation, gender balance, and to the special conditions of developing countries …*" In the end, a total of 26 countries were represented in one or both phases of the AHTEG; 16 of these were considered developing countries or economies in transition[2] as of December 2016 when the Guidance was presented to COP-MOP8. The EU as a "Party" was not represented on the AHTEG, although six of the EU member state countries were Party members on the AHTEG over the two phases (**Table 1**).

The relevance of the Party nominee's expertise "*on the issues relevant for the mandate of the group*" was assessed by the CBD Executive Secretary in order to select these AHTEG members (see **Box 4**). Although the AHTEG members were clearly valued as experts in their fields by the national focal points by who they were nominated, this did not necessarily equate with experience conducting actual cases of risk assessment with LMOs in their countries. In fact, only a small subset of the Party countries have conducted risk assessments for commercial production (see **Table 2** for a list), which could be considered an indication of a Party's "experience" with ERA most like the risk assessment called for in the Cartagena Protocol. ("Commercial Production" is the terminology used here, as it is in the third national reports[3] on implementation of the Protocol, to distinguish the scope of the risk assessment from Field Trials; Contained Use; Food; Feed; Processing. This may also be referred to as "for cultivation" as in the ISAAA database, among other terminology such as "deregulation" or "general release" used in some countries[4].) In fact, many countries that are Party to the Protocol do not yet have biosafety frameworks in place to regulate biotechnology.

If experience with approvals for commercial production is an indication of a Parties' experience with actual cases of risk assessment, then much of this expertise may have been missing among the Party members of the AHTEG. By the time the Guidance was presented at COP-MOP8, 31 countries in the world (26 Parties; five non-Parties) and the EU had approved crops for commercial production, i.e., "for cultivation" according to the ISAAA database on GM (genetically modified) crop approvals. Since then, three more countries (Ethiopia, Nigeria, Swaziland) have also approved a crop for commercial production. **Table 2** shows these 34 countries and the EU, and the crops that have been approved in each. Most of these countries fall into the category of a "developing country"[2]. Of the 26 Party countries that had approved GM crops for commercial production, twelve were represented on the AHTEG; fourteen countries that had approved GM crops for commercial production were not represented on the AHTEG, as shown in **Table 2**.

The number of different crops approved by each country represented on the AHTEG is also shown in **Table 1**. Of the Parties represented on the AHTEG, 12 had approved one or more crop, and eight had approved none. This does not include the six EU member states represented on the AHTEG; although the EU is listed in the ISAAA database for three crop approvals (**Table 2**), the database does not bring up any approvals by individual member states. This is because approvals for commercial production are made at the level of the EU following a review by the European Food Safety Authority (EFSA), and not by individual member states; Therefore, the level of experience with actual cases of risk assessment varies among experts from individual EU member states, including the six EU experts that were Party members of the AHTEG.

Observer Members of the AHTEG

In addition to the "Party" expertise on the AHTEG, experts nominated by non-Party governments were also selected to participate in the AHTEG as "observers," as were experts from other relevant organizations including industry, academia, and other non-government organizations (NGOs) (see **Table 1**). The description of an AHTEG in paragraph 18(a) from the consolidated modus operandi of the SBSTTA (**Box 3**) does indicate that an AHTEG should draw on knowledge and competence from Party experts, as well as experts in the field from '*international, regional and national organizations, including non-governmental organizations and the scientific community, as well as indigenous and local community organizations and the private sector.*' Paragraph 18 of the modus operandi of the SSBTTA does not specify the level of participation from observers, nor does it refer to the rules of procedure for meetings of the Conference of the Parties to the CBD.

However, in the case of the AHTEG on Risk Assessment and Risk Management, and in the open ended online forum, language was included to clearly specify, in the terms of reference in the annex of Decision BSIV/11[1] and as a request to the Executive Secretary in the main text of Decision BSVI/12[1] (see **Box 4**), that participation of observers would be in accordance with the "rules of procedure" for meetings of the Conference of the Parties to the Convention on Biological Diversity and its protocols. (The "rules of procedure" can be found on the Convention on Biological Diversity website: www.cbd.int/convention/rules.shtml). According to the "rules of procedure": *observers* [represented at meetings of the Conference

[2]All countries identified as 'developing countries' throughout this paper are listed as 'developing economies' or 'economies in transition' according to the World Economic Situation and Prospects of the United Nations (United Nations, 2018). This is presumably the list used by the CBD secretariat to identify 'developing countries' in its analyses and reports. More than three quarters of the Parties to the Protocol could be considered developing countries according to this list.

[3]Third national reports on implementation of the Protocol were submitted by 128 Parties to the Executive Secretary of the CBD before MOP8 (in December of 2016). Copies of all third national reports as submitted are available on the Biosafety Clearing House[1].

[4]The Protocol uses the terminology 'intentional introduction into the environment', but does not clearly distinguish introduction for confined field trials from introduction for commercial production (which was incidentally one consistent point of disagreement related to the purpose and scope of the Guidance).

TABLE 1 | Composition of the AHTEG on risk assessment and risk management.

Region/party		AHTEG 1		AHTEG 2		
		MOP4-MOP5 2008-2010	MOP5-MOP6 2010-2012	MOP6-MOP7 2012-2014	MOP7-MOP8 2014-2016	No. of crops approved for cultivation[+]
AFRICA						
Egypt/Mauritania*	D	x	x	x	x	1
Nigeria (1)	D	x	x			0
Nigeria (2)	D	x	x			-
Niger	D	x	x			0
South Africa	D			x	x	3
Zimbabwe	D			x	x	0
Kenya	D				x	0
ASIA &PACIFIC						
China#	D	x	x	x	x	8
Malaysia (1)	D	x	x	x	x	1
Malaysia (2)	D	x	x			-
Japan		x	x	x	x	10
India	D				x	1
EASTERN EUROPE						
Croatia (EU)		x	x	x	x	#
Republic of Maldova	D	x	x	x	x	0
Slovenia (EU)		x	x		x	#
Belarus	D			x	x	0
LATIN AMERICA & CARIBBEAN						
Brazil	D	x	x			4
Cuba	D	x	x			1
Mexico#	D	x	x	x	x	3
Colombia	D			x	x	5
Honduras	D			x	x	1
Antigua & Barbuda					x	0
WESTERN EUROPE & OTHERS						
Austria (EU)		x	x	x	x	#
Germany (EU)		x	x	x		#
Netherlands (EU)		x	x			#
Norway		x	x		x	1
Finland (EU)				x	x	#
New Zealand					x	0
OTHER (NON-PARTY) GOVERNMENTS (OBSERVERS)						
Australia		x	x	x	x	4
Canada		x	x	x	x	8
United States		x	x			17
Argentina				x	x	5
OTHER ORGANIZATIONS (OBSERVERS)						
Bayer Crop Science		x	x	x	x	
Monsanto Company		x	x	x	x	
University of Canterbury		x	x	x	x	
Institute de Estudios Ecologists		x	x			
Federation of German Scientists		x	x			
Public Research & Regulation Initiative		x	x			
College of the Atlantic				x	x	
Flinders University				x	x	
University of Minnesota				x	x	

"D" denotes developing country or economy in transition status according to United Nations (2018)[2].

**The same expert individual was nominated by Egypt for the first AHTEG and by Mauritania for the second AHTEG.*

*[+]No. of different crops approved according to the ISAAA GM Crop Approval Database (see **Table 2**) as of COP-MOP8 in 2016.*

*[#]The ISAAA database lists 3 crops approved by the EU (see **Table 2**), not by the individual EU member states.*

Box 3 | Consolidated modus operandi of the subsidiary body on scientific, technical and technological advice of the convention on biological diversity, paragraph 18 a,b, and e.

Description of *ad hoc* technical expert groups

18. A limited number of ad hoc technical expert groups on specific priority issues on the programme of work of the Conference of the Parties may be established under the guidance of the Conference of the Parties, as required, for a limited duration, to provide scientific and technical advice and assessments. The establishment of such ad hoc technical expert groups would be guided by the following elements:

(a) The ad hoc technical expert groups should draw on the existing knowledge and competence available within, and liaise with as appropriate, international, regional and national organizations, including non-governmental organizations and the scientific community, as well as indigenous and local community organizations and the private sector, in fields relevant to this Convention;

(b) The Executive Secretary, in consultation with the Bureau of the Subsidiary Body on Scientific, Technical and Technological Advice, will select scientific and technical experts from the nominations submitted by Parties for each ad hoc technical expert group. The ad hoc technical expert groups shall be composed of no more than fifteen experts nominated by Parties competent in the relevant field of expertise, with due regard to geographical representation, gender balance and to the special conditions of developing countries, in particular the least-developed and small island developing States, and countries with economies in transition, as well as a limited number of experts from relevant organizations, depending on the subject matter. The number of experts from organizations shall not exceed the number of experts nominated by Parties;

(e) Reports produced by the ad hoc technical expert groups should, as a general rule, be submitted for peer review;

Box 4 | Annex of decision BS-IV/11 on risk assessment, paragraph 1a-b.

Modality of work and the terms of reference for the AHTEG on RA&RM

1. The ad hoc Technical Expert Group (AHTEG) on Risk Assessment and Risk Management shall:

(a) Include experts selected on the basis of their expertise on the issues relevant for the mandate of the Group, based on a standardized common format for submission of CVs from experts nominated by Parties, respecting geographical representation, in accordance with the consolidated modus operandi of the SBSTTA of the Convention on Biological Diversity (decision VIII/10 of the Conference of the Parties, annex III);

(b) Include observers in accordance with the rules of procedure for meetings of the Conference of the Parties serving as the meeting of the Parties to the Protocol.

Decision BS-VI/12 on Risk Assessment, Paragraph 8a-c

Request to the Executive Secretary

8. Requests the Executive Secretary to:

(a) With a view to achieving a balance of current and new members, select experts for the new AHTEG, in consultation with the Bureau of the Conference of the Parties serving as the meeting of the Parties to the Protocol, in accordance with paragraph 18 of the consolidated modus operandi of the Subsidiary Body on Scientific, Technical and Technological Advice of the Convention on Biological Diversity (decision VIII/10, annex III);

(b) Invite other Governments and relevant international organizations to participate in the open-ended online forum;

(c) Ensure that the participation of experts nominated by other Governments and relevant organizations to the open ended online forum and AHTEG is in accordance with rules 6 and 7 of the rules of procedure for meetings of the Conference of the Parties serving as the meeting of the Parties to the Protocol;

of the Party] *may participate without the right to vote in the proceedings of any meeting in matters of direct concern to the body or agency they represent …'* In extending this to members of an AHTEG, this meant that experts from non-Parties and other observers were allowed to attend the face-to-face meetings of the AHTEG and participate in those discussions, as were non-Party and others allowed to contribute posts to the online forum, but these observers did not participate in discussions or decisions on recommendations of the expert group to the COP-MOP. In the case of the AHTEG, observers were at times not even allowed to listen to the discussion on the recommendations among the Party members of the AHTEG.

The reference to the "rules of procedure" in the decisions by the COP-MOP referred to above, which clearly limits participation of non-Party and other experts in the case of this particular AHTEG, may have been considered important by some in order to prevent a conflict of interest, for any purpose, by perceived non-Party proponents or antagonists of biotechnology. At the same time, it almost certainly also limited the AHTEG's ability to develop practical and useful guidance taking into account past and present experiences with LMOs. Most of the global experience with risk assessment of LMOs can be found among the "observers," including the non-Party governments that have issued the vast majority of the approvals for biotech products (**Table 2**). Although the US signed but did not ratify the Convention on Biological Diversity and therefore cannot be a party to the Protocol, as the leading adopter of GM crop applications of biotechnology, the US has participated to the full extent possible as an "observer" in the discussions under the Protocol since the earliest negotiations, as have Canada, Australia, and Argentina, which are also not Party to the Protocol.

The Open-Ended Online Forum

The open-ended online forum on risk assessment and risk management was meant to ensure that multiple experts from Party and non-Party countries, and other organizations could contribute to the discussion and be used by the AHTEG in their deliberations, but this also had limitations, including the restrictions of the rules of procedure (see the text from Decision BS-VI/12[1] paragraph 8c in **Box 4**). The list of registered online forum participants and all of the online forum discussions can be found in their

TABLE 2 | Countries with crops approved for cultivation, and the crops approved in each (Taken from the ISAAA GM Approval Database[a]).

			Crops approved for cultivation	
			As of COP-MOP8 in December 2016	**After COP-MOP8 in December 2016**
PARTY				
	D	Bangladesh	Eggplant	
A	D	Bolivia	Soybean	
A	D	Brazil	Bean, cotton, maize, soybean	Eucalyptus, sugarcane
	D	Burkina Faso	Cotton	
A	D	China	Cotton, maize, papaya, petunia, poplar, rice, sweet pepper, tomato	
A	D	Colombia	Carnation, cotton, maize, rose, soybean	
	D	Costa Rica	Cotton, soybean	
	D	Cuba	Maize	
A	D	Egypt	Maize	
	D	Ethiopia		Cotton
A		European Union	Carnation, maize, potato	
A	D	Honduras	Maize	
A	D	India	Cotton	
	D	Indonesia	Sugarcane	
	D	Iran	Rice	
A		Japan	Alfalfa, canola, carnation, cotton, maize, papaya, rice, rose, soybean, sugarbeet	
A	D	Malaysia	Carnation	
A	D	Mexico	Alfalfa, cotton, soybean	
	D	Myanmar	Cotton	
A	D	Nigeria		Cotton
		Norway	Carnation	
	D	Pakistan	Cotton	Maize
	D	Panama	Maize	
	D	Paraguay	Cotton, maize, soybean	
	D	Philippines	Maize	
A	D	South Africa	Cotton, maize, soybean	
	D	Sudan	Cotton	
	D	Swaziland		Cotton
	D	Uruguay	Maize, soybean	
	D	Vietnam	Maize	
OTHER (NON-PARTY) GOVERNMENTS				
O	D	Argentina	Cotton, maize, soybean	alfalfa, potato
O		Australia	Canola, carnations, cotton, rose,	safflower
O		Canada	Alfalfa, apple, canola, flax, maize, potato, soybean, sugarbeet	
	D	Chile	Canola, maize, soybean	
O		United States	Alfalfa, apple, canola, chicory, cotton, flax, maize, papaya, plum, potato, rice, rose, soybean, squash, sugarbeet, tobacco, tomato	creeping bentgrass,

[a] *http://www.isaaa.org/gmapprovaldatabase/default.asp as of Sept. 29, 2018.*

"D" denotes developing country status according to United Nations (2018)[2].

"A" denotes a Party member and "O" denotes observer member of the AHTEG on Risk Assessment and Risk Management.

entirety on the Biosafety Clearing House[1]. There were ∼300 individuals enrolled in the online forum with a wide range of expertise, although a much smaller number of these individuals regularly participated in any given forum discussion; ∼75% of those enrolled were individuals nominated by Parties, ∼10% nominated by non-Party governments, and the rest from "other organizations."

An example of the online forum participation comes from the last online forum discussion that took place before COP-MOP8 (April 25-May 9 2016). The topic of this discussion was "Feedback on the Proposed Revisions to the Guidance." This was an important online forum discussion because it was the only opportunity to provide feedback by individuals not on the AHTEG, to the AHTEG's proposed revisions based on

the comments from the testing of the Guidance (discussed in more detail later). Instead, the feedback requested in this forum discussion was strictly limited to certain revisions and comments on the whole document were not invited. The discussion was open for 2 weeks (which was typical), with six posts coming in the first week and 48 coming in the second week. These 54 posts came from 29 individuals: 14 nominated from eight Parties (two who were members of the AHTEG), three nominated from three non-Parties, and 12 nominated from ten other organizations, including several who were members of the AHTEG. While a number of posts in this forum discussion were supportive of the Guidance, a number also shared frustration with the limited ability the forum presented for input on the Guidance.

The online forum was commended by the COP-MOP in its COP-MOP5 decision (Decision BSV/12[1]) as an innovative method and efficient means to maximize the use of limited resources. It did provide an opportunity for participation by a large group of experts with broad and diverse backgrounds and experiences, with varying motivations to participate, including the non-Party governments, the biotech industry, academics, and non-government organizations, some with clear pro- or anti-biotech agendas. However, the requests for input in the online forum over the years were generally narrowly limited to specific points determined by the CBD secretariat, and although this may have been necessary for the functioning of the forum, it was not clear how the input from the online forum on these specific points was ultimately used, by the AHTEG or in other ways, to shape the Guidance. Although the online forum was a good idea in theory and did provide an opportunity for more experts to voice an opinion, in practice it did not offer an effective tool to develop or improve the Guidance.

Weighing Expert Input vs. Party Input

It was not always clear whether the members of the AHTEG or Online Forum participants, from Parties or others, were meant to be contributing to the discussions based on their own experiences as experts with risk assessment, or on behalf of the political positions of the governments or organizations that nominated them. In the latter case, particularly in following the "rules of procedure" of the Convention, the discussions were bound to and did become more like the negotiations of the Parties and less like an expert consultation. It would seem from the description of an AHTEG in the consolidated modus operandi of the SBSTTA (paragraph 18(a) in **Box 3**), that an AHTEG should be seeking "expert" input, rather than "Party" input. Yet, the discussions of this AHTEG and the online forum often appeared to be "Party"-driven, rather than "expert"-driven, with a tally of Party vs. other expert opinions on each side of an issue.

In the final deliberation, after an exhausting eight years, the "Party members" of the AHTEG, without the "observer members" of the AHTEG which was according to the "rules of procedure" as specified in the COP-MOP decisions (**Box 4**), "unanimously" agreed to recommend endorsement of the Guidance to the COP-MOP (UNEP/CBD/BS/COP-MOP/8/INF/2[1]). It was made clear at COP-MOP8, however, that a decision by the "Party members" of the AHTEG was not the same as the decision by the Parties at the COP-MOP. Although the priority to Parties on the AHTEG meets with the rules of procedure of meetings of the Convention and its protocols, it does not clearly align with the role of an AHTEG as set forth in the consolidated modus operandi of the SBSTTA, and it was apparently not an effective means to develop technical guidance based on expert input. It is always a challenge to separate political discussions from technical issues in risk assessment and regulation of biotechnology (Hokanson et al., 2018). The experience with this AHTEG further demonstrates what should be obvious, that it is not practical, if even possible, to "negotiate" the contents of a technical guidance document.

MISGUIDED TESTING OF THE GUIDANCE

The Testing Process

More difficulties for the development of the Guidance were encountered in the testing that was conducted between COP-MOP6 and COP-MOP8. When the first draft of the Guidance was presented at COP-MOP5 the Parties called for '*further scientific reviewing and testing to establish its overall utility and applicability*' (Decision BSV/12[1]). In response, after COP-MOP5 there was a further round of revisions by the AHTEG and the online forum. At COP-MOP6, the Parties commended the progress on the Guidance, and called for the Guidance to be '*tested nationally and regionally for further improvement in actual cases of risk assessment and in the context of the* [Protocol]' (Decision BSVI/12[1]). In response after COP-MOP6, the first AHTEG was brought to a close, and a reconstituted AHTEG was established (see **Figure 1** and **Table 1**), and Parties, other Governments, and other organizations were encouraged '*through their risk assessors and other experts who are actively involved in risk assessment, to test the Guidance in actual cases of risk assessment*' and submit the results to the Biosafety Clearing House.

A notification for the testing of the Guidance (SCBD/BS/CG/MPM/DA/82041[1]) described the process for the testing in broad terms, including the use of a specified form ('*The Questionnaire for Reporting Results of the Testing of the Guidance on Risk Assessment of Living Modified Organisms*'[1]). The methodology to employ for conducting the test "in actual cases of risk assessment" was not specified beyond a recommendation to identify an "actual case" to consider. There was no recommendation on how "risk assessors" should be identified, and a description of the credentials of the testers or description of the testing methodology employed by the testers was not requested with the submissions. Thus, it was such that tests were apparently conducted in any number of undefined, different ways. The form simply asked the testers to rate the six parts of the Guidance (**Box 2**) on a scale from 1 (Strongly Agree) to 5 (Strongly Disagree) for each of four criteria: (1) "practical," (2) "useful," (3) "consistent with the protocol," and (4) "takes into account past and present experiences with LMOs." For each of the sections rated there was also a space to suggest specific improvements, and a space at the end of

the questionnaire to "*provide additional feedback regarding the testing of the Guidance*."

The Results of the Testing

The '*individual submissions*' (filled questionnaires) from all of the participants in the testing can be found on the Biosafety Clearing House[1], and a report on the results of the testing was shared at COP-MOP7 (UNEP/CBD/BS/COP-MOP/7/INF/3[1]). Forty-three of the 171 Parties to the Protocol (25% of all Parties), three non-Party governments, and ten 'other organizations' participated in the testing (see **Tables 3A,B**). All of these participants tested the Roadmap section (Part I) of the Guidance (see **Box 2**); many participants submitted test results on the Roadmap only, while the other sections were tested only by some and not by others. Of the 43 Parties that participated, 28 are considered "developing countries,"[2] as described in the report on the results.

The participation of these developing country Parties held significance in the Secretariat's analysis of the results, presumably because the Protocol (in Article 22) calls for capacity building in biosafety for the purpose of effective implementation of the Protocol in developing country Parties and in Parties with economies in transition. The Strategic Plan for the Cartagena Protocol for the period 2011–2020 (Decision BS-V/16, Annex I), coincidentally agreed to by the COP-MOP after the work of the AHTEG had begun, includes risk assessment and risk management as part of its capacity building objectives, and indicators to measure progress include measures of Parties that are using the developed technical guidance and that are of the opinion that the technical guidance is sufficient and effective. Thus, it seemed the Secretariat viewed "developing countries" that are the target of capacity building as an important group for which to measure the level of agreement with the criteria in the testing.

In the report, the results are shown in a bar graph as the '*overall level of agreement that* [the Guidance] *is practical, useful, consistent with the Protocol, and takes into account the past and present experiences with LMOs*' averaged across the ratings for the four distinct criteria within certain groupings (i.e., All Parties, Developing Country Parties, Other Governments, and Organizations). A series of graphs also showed these groupings for each of the criteria independently and for the different sections of the Guidance. All of those graphs show that average scores from the developing country parties were equal to or slightly higher than from all Parties, and both of these groups' scores were considerably higher than the average score from the three non-Party governments that participated in the testing. Although an interpretation of these results as reported to COP-MOP7 may arguably not be particularly meaningful, if interpreted as a measure of the level of "agreement" that the Guidance meets the criteria, the relative scores among these groups could be an indication that the developing country Parties "agree" the most that the Guidance is "practical," "useful," "consistent with the Protocol," and "takes into account past and present experience." Likewise, it could be surmised that non-Party governments "agree" the least.

Yet, relative scores among other groupings not considered as part of the report to COP-MOP7, could indicate something different. Most important is the notable difference between ratings provided by countries (Party, Non-Party, or Developing) that have experience with conducting risk assessments and those that don't. **Figure 2** shows the number scores for the testing, specifically on the Roadmap section of the Guidance (here the focus is on the Roadmap because it is the core of the Guidance and the section tested by all participants) for additional groupings averaged across all four criteria. This includes three of the groupings included in the report (All Parties, Developing Country Parties, Non-Party Governments), and four additional groupings. These are "Parties that have conducted risk assessments" for commercial production and "Parties that have not conducted risk assessments" for commercial production (many of these do not yet have biosafety frameworks) according to the third national reports[3,4]. Developing countries can be further grouped into "Developing Country Parties that have" or "Developing Countries that have not" conducted risk assessments for commercial production. **Figure 2** demonstrates that there is more disparity between the higher average score from Parties who *have not* conducted risk assessments for commercial production (4.1) and the lower score from Parties who *have* conducted these risk assessments (3.6), and even more so between developing country parties who *have not* conducted these risk assessments (4.3), and those who *have* (3.1).

These trends from the results based on the number scores of the testing seem to indicate that Parties, including developing country Parties, who have more experience with risk assessment, rated the Roadmap lower across all criteria than did those with less experience. If this is the case, it stands to reason that the non-Party governments, who presumably have the most experience conducting risk assessments on LMOs, agreed the least that the testing met the criteria of "useful," "practical," "consistent with the Protocol," and "takes into account past and present experience with LMOs." The relatively lower scores among Parties who have experience compared to those with less experience may tell us more about the utility and applicability of the Roadmap than does the relative score of developing countries compared to all Parties that was central in the analysis from the Executive Secretary in the report to COP-MOP7. Regardless, these number scores can only tell us how the testers rated the Guidance against the testing criteria, and tell us very little about the utility and applicability of the Guidance in '*actual cases of risk assessment*,' which was the stated objective of the testing according to Decision BSVI/12[1].

The Revisions Based on the Testing

However the trends are interpreted, it would be imprudent to only consider the number scores as an indication of the practicality or usefulness of the Guidance. At COP-MOP7, where the report from the testing (UNEP/CBD/BS/COP-MOP/7/INF/3[1]) was presented to the Parties, it was decided that the Guidance should still be revised and improved '*on the basis of the feedback provided through the testing with a view to having an improved version of the Guidance by MOP8*' (Decision BS-VII/12[1]). More than 775 comments were submitted by participants in the testing on the questionnaires along with

TABLE 3A | Results from participants in the testing of the Roadmap (Part I) of the 'Guidance on Risk Assessment of LMOs developed by the AHTEG under the Cartagena Protocol on Biosafety, and the answers by the Party countries to questions on risk assessment (see **Box 6**) in the third national reports3 on implementation of the Protocol, by countries that are currently conducting risk assessments for commercial production.

Q89. YES—Conducting any risk assessments					
Q90. YES—Conducting Risk Assessments for Commercial Production					
		Q85a. Using any guidance	**Q86. Using AHTEG guidance**	**Roadmap testing results**	
				Avg. score all criteria	**Number of comments**
Party					
Norway		Yes	Yes	5	5
Cuba	D	Yes	Yes	4.75	2
Austria (EU)		Yes	No	4.75	15
Egypt	D			4.75	4
Czech Republic (EU)		Yes	Yes	4.5	7
Slovenia (EU)		Yes	No	4.25	0
Spain (EU)		Yes	Yes	4.25	1
Costa Rica	D	Yes	Yes	4	4
European Union*		Yes	No	4	7
Germany (EU)		Yes	No	4	21
Portugal (EU)		Yes	No	4	0
Mexico	D	Yes	No	4	19
South Africa	D	Yes	No	4	12
Italy (EU)		Yes	No	3.75	1
Uruguay	D	Yes	No	3.75	2
VietNam	D	Yes	No	3.5	1
Belgium (EU)		Yes	No	3	5
Colombia	D	Yes	No	2.5	11
Netherlands (EU)		Yes	No	2.25	7
India	D	Yes	No	2.25	48
Brazil	D	Yes	No	2	27
Honduras	D	Yes	No	2	6
Japan		Yes	No	2	25
Philippines	D	Yes	No	2	1
Average				3.55	9.6
Other (Non-Party) Governments					
Canada				2.5	21
United States				2.25	10
Australia				2	50
Average				2.13	27

"D" denotes developing country or economy in transition status according to the United Nations (2018)[2].
**The European Union participates in the COP-MOPs as a Party, as do the individual member states.*

the ratings; 488 of these comments were on the Roadmap (Part I) of the Guidance alone. The numbers of comments submitted by the participants on the Roadmap section only are shown in **Tables 3A,B**, and it should be noted that in general participants who gave the Roadmap (as with the Guidance) lower scores than those who gave higher scores also submitted more comments.

In the decision from COP-MOP7, the Parties established a mechanism for the AHTEG to revise and improve the Guidance on the basis of the feedback, as described in some detail in paragraph 1 of the terms of reference for the online forum and AHTEG in the Annex to Decision BS-VII/12[1] (see **Box 5**). However, the "streamlining" of the comments outlined in paragraph 1(c) of the methodology was not done by the AHTEG as described, but by a subgroup of five AHTEG members (experts nominated from China, Finland, Mexico, Republic of Moldova, and Zimbabwe, selected from the AHTEG at its last face-to-face meeting *before* COP-MOP7 (BS/COP-MOP/7/10/Add.2[1]). This subgroup decided which comments would be "taken on board." A record of the "Subgroup Discussions (2014–2016)" can be found on the Biosafety Clearing House[1], and a document with the justifications for the actions taken by the subgroup on every comment submitted in the testing of the Guidance was provided to COP-MOP8 (BS/COP-MOP/8/INF4[1]).

TABLE 3B | Results from participants in the testing of the Roadmap (Part I) of the "Guidance on Risk Assessment of LMOs" developed by the AHTEG under the Cartagena Protocol on Biosafety, and the answers by the Party countries to questions on risk assessment (see **Box 6**) in the third national reports[3] on implementation of the Protocol, by countries that are currently not conducting risk assessments for commercial production or not conducting any risk assessments.

Q89. YES—Conducting any risk assessments **Q90. NO—Conducting Risk Assessments for Commercial Production**		**Q85a. Using any guidance**	**Q86. Using AHTEG guidance**	**Roadmap testing results**	
				Avg. score all criteria	**Number of comments**
Party					
Bolivia	D	No	No	4.5	13
Belarus	D	Yes	Yes	4.5	18
Denmark (EU)		Yes	No	4	3
Turkey	D	No	No	3.5	2
New Zealand		Yes	Yes	2.25	2
Malaysia	D	Yes	Yes	2.25	25
El Salvador	D	Yes	Yes	2	19
Q89. No—Not Conducting Any Risk Assessments					
Libya*	D	–	–	5	0
Mauritania	D	No	No	5	0
Niger	D	No	No	5	0
Republic of Moldova	D	No	Yes	5	5
Syria*	D	–	–	5	0
Tajikistan	D	No	No	5	1
Yemen	D	No	Yes	5	0
Liberia	D	No	No	4.75	1
Bosnia	D	Yes	No	4.25	0
Hungary (EU)		Yes	No	4.25	5
Georgia	D	Yes	No	4	0
Peru	D	No	No	3.5	15
Average				4.14	5.7
Other Organizations					
Eco-Tiras				5	6
GenOk-Center for Biosafety				5	4
Int'l Association for Human&Animal Health Improvement				5	0
State University Maldova				5	4
Third World Network				5	8
Academy of Science Maldova				4.75	5
ENCA EPA				4.75	9
Friends of the Earth-Ukraine				4.5	3
Public Research & Regulation Initiative				2	16
Global Industry Coalition				1.75	5
Average				4.275	6.7

"D" denotes developing country or economy in transition status according to the United Nations (2018)[2].
** Did not submit third national reports by the time of COP-MOP8.*

Although the work of the subgroup was completely transparent by making their assessments and justifications available for viewing, it would be incorrect to assume that these justifications were the work of the entire AHTEG and the Online Forum. Neither the AHTEG nor the Online Forum had an opportunity to discuss many of the decisions by the subgroup about whether or not to "take comments on board." This process did result in numerous and significant changes to the Guidance by the time it was presented at COP-MOP8 for the Parties to consider, although many of the concerns raised in the feedback to the testing were still not addressed in the most recent revised version. At COP-MOP8, unfortunately, a number of Parties did not feel there had been an opportunity to consider whether these changes resulted in an "improved" guidance, resolving some of the more serious concerns with the Guidance that had been expressed over the years in the on-line forum and AHTEG discussions, or as a result of and in the comments from the testing. The outcome after this lengthy and arduous process employed to test the Guidance and revise it based on the results of the testing

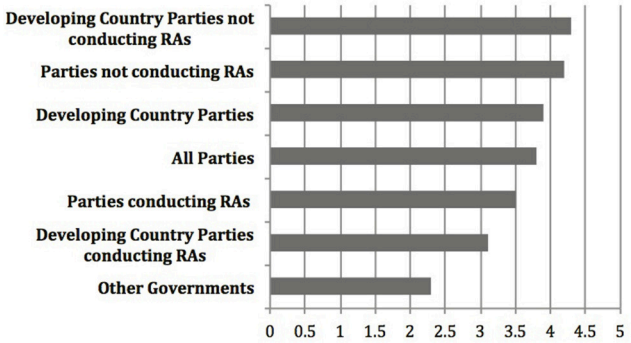

FIGURE 2 | Overall level of agreement that the Roadmap is practical, useful, consistent with the Cartagena Protocol on Biosafety, and takes into account past and present experience with LMOs, based on the results of the testing of the Guidance as gathered by the CBD Secretariat, where 1 is strongly disagree and 5 is strongly agree (The results of the testing can be found at https://bch.cbd.int/protocol/testing_guidance_RA.shtml).

Box 6 | Third national report on implementation of the Cartagena Protocol on Biosafety.

Relevant questions on risk assessment

Q. 85 Has your country adopted or used any guidance documents for the purpose of conducting risk assessment or risk management, or for evaluating risk assessment reports submitted by notifiers? a. Risk Assessment. b. Risk Management.
Q. 86 Is your country using the "Guidance on Risk Assessment of LMOs" (developed by the Online Forum and the AHTEG on Risk Assessment and Risk Management) for conducting risk assessment or risk management, or for evaluating risk assessment reports submitted by notifiers?
Q. 89 Has your country ever conducted a risk assessment of an LMO including any type of risk assessment of LMOs, e.g., for contained use, field trials, commercial purposes, direct use as food, feed, or for processing?
Q. 90 If you answered Yes to question 89, please indicate the scope of the risk assessments (select all that apply): Commercial Production; Field Trials; Contained Use; Food; Feed; Processing.

Box 5 | Annex of decision BS-VII/12, paragraph 1a-d.

AHTEG mechanism to improve and revise the Guidance

1.Taking into account the results of the testing process, established in decision BS-VI/12, the Guidance on Risk Assessment of LMOs shall be revised and improve in accordance with the following mechanism:

(a)After the seventh meeting of the COP-MOP, the Secretariat will group the original comments provided through the testing of the Guidance. The grouping will be done in the form of a matrix based on the following categories: statements that do not trigger changes; editorial and translational changes; suggestions for changes without a specified location in the Guidance; and suggestions for changes to specific sections of the Guidance (sorted by line numbers);
(b)The AHTEG shall review the grouping of comments done by the Secretariat and work on the suggestions for changes;
(c)The AHTEG shall streamline the comments by identifying which suggestions may be taken on board and providing justification for those suggestions that may not be taken on board. The AHTEG will also provide concrete text proposals for the suggestions to be taken on board with a justification where the original suggestion was modified;
(d)The Open-ended Online Forum and the AHTEG shall subsequently review all comments and suggestions with a view to having an improved version of the Guidance for consideration by the COP-MOP at its eighth meeting.

seems to indicate that unfortunately the testing process missed its mark.

A CLOSER LOOK AT EXPERIENCE IN RELATION TO THE GUIDANCE

Experience Based on the Third National Reports

The third national reports[3] submitted by the Parties on the implementation of the Protocol shed even more light on the relationship between experience with risk assessment and the development and testing of the Guidance. The reports included answers to questions regarding risk assessment of LMOs in relation to the use of guidance, including "the Guidance." (The relevant questions related to risk assessment and the Guidance are shown in **Box 6**). In the official meeting document on Risk Assessment and Risk Management (UNEP/CBD/BS/COP-MOP/8/8[1]) prepared for COP-MOP8, the CBD Secretariat reported some select information from these third national reports to suggest that the Guidance is being used or is useful. With regard to the answers to the third national reports aligned with the results of the testing of the Guidance, of the Parties that have conducted *any* risk assessments, 31 also participated in the "Testing" of the Guidance; Of those 31 Parties, 60% of these "agreed-4" or "strongly agreed-5" that the Guidance "is useful" in response to the testing, which might suggest that 60% of these Parties do consider the Guidance useful, as implied in the above referenced COP-MOP8 meeting document from the Secretariat. The average "agreement rating" among the 31 Parties was 3.4 that the Guidance is "useful or has utility."

Upon a closer look at the third national reports, it can also be noted that, of the Parties that indicated having NOT conducted Risk Assessments, only ten also participated in the testing of the Guidance. Of those ten, all (100%) "agreed" or "strongly agreed" that the Guidance "is useful," with an average agreement rating of 4.7, again demonstrating that Parties with less experience conducting risk assessments rated the Guidance higher than Parties with more experience. The third national reports also show that of those Parties that have conducted *any* type of Risk Assessment, 89% also reported having adopted or used *any* guidance documents for the purpose of conducting or evaluating risk assessments; and of those Parties that have adopted or used *any* guidance, 74% reported *not* using "the Guidance," indicating that there is some other guidance that they are using.

Tables 3A,B show the answers to the questions on risk assessment from the third national reports against the testing scores on the Roadmap section of the Guidance. **Table 3A** includes the Parties that indicated they are conducting risk assessments when the scope of the assessment was for commercial production, and **Table 3B** includes those parties that have conducted risk assessments *not* for commercial production, or have not conducted any type of risk assessments. The testing scores for the Roadmap section of the Guidance, averaged across the four criteria, for all of the Parties and the non-Party governments who participated in the testing are shown in **Tables 3A,B**, along with the number of comments provided by each participant on the Roadmap section. **Tables 3A,B** also show the answers to the question from the third national reports asking whether the Party is using *any* guidance for risk assessment (Q85a), and whether the Party is using the Guidance developed by the AHTEG for conducting risk assessments (Q86) (see **Box 6**). In addition to the Parties shown in **Tables 3A,B**, 22 more Parties (not included in the tables because these did not participate in the testing) also reported in their third national reports using any guidance for risk assessment and not using the AHTEG Guidance[5]. The EU and all of the EU member states that submitted third national reports indicated that they are using guidance for risk assessment, and not using the AHTEG Guidance. This is predictable because the EU has a well-developed existing guidance for ERA of genetically modified plants (EFSA, 2010).

These trends from the third national reports against the results of the testing do seem to indicate that most Parties (developed or developing) that have conducted risk assessments are, in fact, *not* using the Guidance and agree less that the Guidance is "useful," as for all other criteria for the testing, than Parties that have not conducted risk assessments. Most of these Parties that have conducted risk assessments have adopted and/or used other guidance documents for the purpose of conducting risk assessment rather than the Guidance developed by the AHTEG. Parties that have conducted risk assessments and have followed other guidance may have given lower scores in the testing because they have more experiences upon which to base their evaluation of the Guidance.

It is important to note these trends when considering the "usefulness" of the Guidance. While it stands to reason that Parties with limited experience in risk assessment are more in need of guidance, it also stands that Parties with more experience are in a better position to develop guidance based on that experience. Furthermore, many Parties "with experience" are in fact developing countries, and these developing countries with experience should not be conflated with Parties that are more in need of guidance. Perhaps more consideration of the experiences of Parties with actual cases of risk assessment and the other guidance documents these Parties have adopted and/or used, which seemed to be the original intent for the AHTEG, would have resulted in a more useful guidance document for the less experienced Parties, and one that could have been endorsed by the Parties.

When Experts With Experience Test the Guidance

As it is, many of the "experts" participating in the development of the Guidance, whether from Parties or not, developing countries or not, on the AHTEG, in the online forum, and participating in the testing, although experts in their fields, had limited experience with "actual" risk assessments of LMOs upon which to base their contributions to these discussions. Recognizing this, as there was an open invitation after COP-MOP7 to "*Parties, other Governments, and relevant organizations to test or use, as appropriate, the Guidance in actual cases of risk assessment*" (Decision BSVII/12[1]), a workshop was organized and took place on Feb. 1-3, 2016 in Washington DC[6], bringing together a group of individual experts who have worked with the regulatory authorities in countries that do have experience with "actual cases" of ERA for general release into the environment. The purpose of the workshop was to review the Roadmap from the Guidance at that stage and compare this information to actual cases of risk assessments in their country, noting how these fit into the steps outlined in Annex III of the Protocol (**Box 1**).

Individual experts who participated in the workshop were from the following countries: Argentina, Australia, Brazil, Canada, Colombia, European Union, India, Japan, Mexico, Netherlands, South Africa, and the United States. (Two participants from the US were from the two agencies involved in ERAs with two separate mandates: USDA APHIS and EPA). Individuals from these countries were selected to participate in this testing exercise based mainly on their personal experience as risk assessors in countries (from the list shown in **Table 2**) that had approved for commercial production more than one crop. Eight of the twelve participants had their experience from work in countries that are Parties to the Protocol, and four were from non-Parties (Argentina, Australia, Canada, US); also, five of the participants were from countries that are considered "developing countries" (Argentina, Brazil, Colombia, Mexico, South Africa)[2].

Thus, the participants at this workshop were a good representation of countries with experience in conducting risk assessment, by individuals who had actual experience conducting risk assessments in their countries. None of the experts who participated were at that time members of the AHTEG. Some of the experts who were invited to participate had, during the online forums, expressed some concern about the Guidance as it was being developed, suggesting that what they observed in the Guidance did not align with their experiences with risk assessment. It should also be noted that all opinions shared during this workshop were understood to be that of the

[5]Burkina Faso (D), Bulgaria (D), Cameroon (D), Estonia (EU), Finland (EU), France (EU), Ghana (D), Guatemala (D), Indonesia (D), Lithuania (EU), Malawi (D), Nicaragua (D), Romania (EU), Slovakia (EU), South Korea, Sweden (EU), Switzerland, Tanzania (D), Thailand (D), Uganda (D), United Kingdom (EU), Zimbabwe (D).

[6]The workshop was organized by the University of Minnesota Stakman-Borlaug Center for Sustainable Plant Health and was supported primarily through a grant from the USDA National Institute of Food and Agriculture, Biotechnology Risk Assessment Grant Program (Grant no. 2015-33522-24097 awarded to PI K. Hokanson, University of Minnesota).

individuals based on their experience, and individuals were not asked or expected to represent the position of their government (Party or non-Party) in any way.

In order to conduct this "testing," the experts were provided with a copy of the Roadmap (note, this was the version of the Guidance that was available as AnnexII in the report from the AHTEG (UNEP/CBD/BS/RARM/AHTEG/2015/1/4[1]) after its first meeting after COP-MOP7, in Brasilia, November 2017), and a template that captured all of the concepts in the Roadmap into a table, taken from the text of the Roadmap in the order they appeared there. The experts each chose a recent, actual case of risk assessment from their country to consider as they went through the concepts of the Roadmap to determine whether each concept is considered or not in the ERA that was actually done. This exercise served as a guide for each of the participants to present to the group how their risk assessments compare with the Roadmap. (Participants from Australia and Japan were not able to attend the workshop, but completed the evaluation and shared this for the discussion during the workshop).

The Outcome of the Testing by Experts With Experience

At certain points, it was difficult for the participants to say whether the concepts in the Roadmap were considered in their risk assessments. The participants noted that there are concepts in the Roadmap that may be considered during their risk assessment, but are not necessarily captured as part of the document that is finally produced from the ERA, and there were other concepts that were clearly only considered in certain cases. In a few cases, the participants struggled to understand the concept as it was described in the Roadmap. Yet, it is notable that nearly half of the concepts in the Roadmap are ones that most participants agreed are considered as part of their risk assessments, and there were only a few concepts (∼10%) that most participants said they do *not* consider. The remaining concepts, however, were considered by some and not considered by others. This suggests that there is, among the different risk assessments in different countries, much in common, but also certainly much of the Roadmap that does not reflect a common approach among countries. Still, participants in this workshop agreed that many of the concepts captured in the Roadmap are indeed relevant to risk assessment.

Interestingly, the participants who thought that most (∼90%) of the concepts from the Roadmap are considered in their risk assessments came from the EU, the Netherlands, South Africa, Japan, and the US. (In the case of the US, a concept was counted as "considered" if it was considered in risk assessments at either APHIS or EPA). The participants who thought that the least concepts are considered in their risk assessments were India and Argentina, although even these participants thought that more than half of the concepts are considered in their risk assessments. In the middle were Australia, Brazil, Canada, Colombia, and Mexico. There did not appear to be a clear separation between the participants whose experience was with Party governments and non-Party governments, or between the developed and developing countries with regards to the concepts in the Roadmap. This also suggests that experience in conducting risk assessment is more predictive of testers response to the Guidance than the overall economic development of their country or status as a Party. The results of this test also seem to suggest that it is not so much the concepts in the Guidance (or at least the Roadmap), but some other aspects that caused the concerns expressed by Parties at COP-MOP8.

The remainder of the workshop was devoted to discussion to elucidate this distinction, including some time working in smaller groups to consider possible changes to improve the various sections of the Roadmap. The overriding conclusion from these group discussions was that, although many of the concepts are included in their risk assessments, the roadmap simply does not reflect the "process" followed for risk assessment based on their experience. Ultimately, the participants were able to agree on a set of "consensus points" that summarize the major flaws in the Roadmap:

- Many basic concepts presented in the Roadmap are relevant for ERA, but the Roadmap is organized in a way that confounds the risk assessment process.
- The Roadmap does not capture the experience that has been gained in the last 25 years of LMO risk assessment.
- There is a lack of clarity on the objectives and the target audience for the Roadmap.
- The Roadmap is not always consistent with and goes beyond the scope of Annex III of the Cartagena Protocol.
- The level of detail and attention given to certain subjects in the Roadmap is disproportionate in terms of relevance to ERA.
- The treatments of uncertainty and monitoring in the Roadmap, in particular, are not consistent with Annex III, nor based on experience with risk assessment.
- The Roadmap is not appropriate for risk assessments related to field trials.
- Key terms and concepts should be more clearly identified, defined, and linked to existing terminology in general use for ERA, and put into the appropriate context.
- These concerns with the Roadmap should be adequately addressed and agreed upon before any additional guidance is considered.

Most of these same points are also reflected among the comments submitted with the results of the testing and in the online forum discussions. In general the participants of the workshop did not see an easy way to address these flaws through straight-forward revisions or rearrangements in the text. Therefore, the result of this testing led to a conclusion that the Roadmap is not practical or useful as a guide for risk assessment and the problems with it cannot be easily fixed. Although this workshop took place before the final revisions by the AHTEG were presented at COP-MOP8, the problems identified by this expert group remained in the final version.

CONCLUSIONS

The AHTEG completed its mandate to work on the Guidance on Risk Assessment of LMOs by COP-MOP8 in 2016, where the COP-MOP "took note of" the Guidance, but did not endorse

it, calling it "voluntary" Guidance in the decision, making it available but entirely clear that there is no obligation by Parties to use this Guidance. Although the work on the Guidance in its current form is finished, the work on risk assessment under the Cartagena Protocol continues. There was a decision at COP-MOP8 (Decision BSVIII/12[1]) to extend the online forum on Risk Assessment and Risk Management to continue to exchange experiences on risk assessment, provide information and views on perceived gaps in existing guidance materials, and provide proposals to address any gaps identified. In the discussions that ensued, some Parties submitted requests for additional guidance on specific issues while other Parties made a case that no additional guidance is needed at this time, a difference of opinion that had been expressed continuously throughout the process to develop the Guidance.

Therefore, the decision for further work on risk assessment coming out of the most recent COP-MOP9 (CBD/CP/MOP/9/13) which took place in Sharm-el Sheik, Egypt in November of 2018, focuses on developing a "process" to identify and prioritize the specific issues, if any, of risk assessment for consideration by COP-MOP10 before there will be any decision to develop any further guidance. In effect, the request from COP-MOP9 is responding to the fact that, in addition to the development of a dysfunctional Roadmap, development of further guidance on additional topics had already been attempted by the AHTEG on a rather arbitrary list of specific issues, i.e., on stacked genes, abiotic stress, mosquitos, trees, and monitoring, and proposed for fish and synthetic biology, without a clear process in place for selecting these issues. A clear process and criteria for identifying and prioritizing specific issues for developing further guidance on risk assessment is absolutely essential, and one critically important criteria to consider, as described in Annex I of the COP-MOP9 Decision, is whether a topic or issue poses challenges to existing risk assessment frameworks, guidance, and methodologies. Had existing risk assessment approaches been given due consideration before the Guidance was pursued initially, there may have been considerable savings in time, energy and money.

However, if it is decided that further guidance on a specific issue is needed, a more important decision by the COP-MOP will be about the proper process for developing that guidance and how to include the most relevant and appropriate expertise. The COP-MOP must consider whether an AHTEG functioning according to the consolidated modus operandi of the SBSTTA and rules of procedure of the Convention, as was this past AHTEG, is the most appropriate body of experts to develop such guidance. Clearly, the outcome from the work of the AHTEG and online forum on Risk Assessment and Risk Management tells us that something must be changed in this process. At a minimum, the COP-MOP must develop a means of separating political discussions from an undertaking by technical experts. There must be a more effective way a group of experts can develop guidance that represents consensus on a technically sound approach to risk assessment, or a way to capture in the outcome the differences of opinion that might be more meaningful as guidance to Parties, rather than delivering a compromise document.

The experience with the Guidance on Risk Assessment of LMOs, as described herein, seems to indicate that the only way to reach agreement among Parties is not to base any further guidance on what experts think "should be done," but to base it on commonalities from experiences with existing, actual cases of risk assessment. In this case, risk assessment guidance under the Cartagena Protocol could only be developed on specific issues where there is experience with risk assessment and when Parties can agree that the guidance being developed represents their experience. Many Parties have the opinion that many of the specific topics that have been identified to date could be assessed for risk based on an extension of current practices. This includes some applications of synthetic biology, genome-editing, and gene drive systems in living modified organisms, all currently topics of discussion for risk assessment under the Convention and its Protocol. With respect to risk assessment, the many possible applications of these technologies must be considered on a case-by-case basis.

In fact, it is currently and should remain the responsibility of Parties, within their own domestic frameworks, to determine how to do risk assessment that is consistent with Annex III of the Protocol and national environmental policy. Ultimately, Parties with less experience may do better to identify and choose Parties with more experience from which they may learn, in order to develop guidance on risk assessment that meets their specific needs while remaining consistent with their obligations under the Protocol. The COP-MOP may do better to devise ways to support this sort of Party-to-Party assistance, or to invite other less constrained input from experts with experience, rather than putting limited resources into a process that may be correct according to policy, but in practice is destined to fail.

AUTHOR CONTRIBUTIONS

KH collected and analyzed the information and wrote the manuscript based on the documents referred to within, and on personal observation as an observer on the AHTEG for Risk Assessment and Risk Management from 2012 to 2016 and at COP-MOPs 2-9.

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