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## RISK ASSESSMENT NEWS

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### Field-Evolved Resistance in Corn Earworm to Cry Proteins in Transgenic Corn and Its Implications

*P. Dilip Venugopal and Galen P. Dively*

Crops engineered with genes from the soil bacterium *Bacillus thuringiensis* (Bt) to express specific proteins with insecticidal properties (Cry proteins) are a major tool to manage agricultural insect pests. Since commercial introduction in the 1990s, this biotechnology is increasingly adopted in many parts of the world, especially for corn and cotton pest management. In 2015, 81% of all corn planted in the U.S.<sup>1</sup> was engineered genetically with Bt for controlling insects (caterpillars and beetles). Biotechnology enables the engineering of crops to express one or more Bt Cry proteins simultaneously for the same target pests (called pyramided traits). However, with widespread use of genetically engineered crops, insect resistance to these proteins is a major threat to the Bt technology.

Resistance development is particularly a concern for some insects for which the Bt Cry protein is not highly toxic, enabling a few to survive. For Cry-expressing Bt corn, the high dose requirement for resistance management is not achieved for insects such as corn earworm, *Helicoverpa zea* (Boddie), and western corn rootworm, *Diabrotica virgifera virgifera* LeConte, which are more tolerant to the Bt toxins. Recent studies document and characterize the resistance of these insect pests to multiple Cry proteins (both single protein and pyramided traits) expressed in Bt corn, threatening the sustainability of the Bt technology<sup>2,3</sup>. *H. zea* is the key pest of sweet corn. It is polyphagous in many agricultural crop systems and capable of migrating long distances<sup>4</sup>. In this article we discuss factors contributing to the evolution of resistance in corn earworm to transgenic corn expressing Cry1Ab and Cry1A.105/Cry2Ab2 proteins. Further, we describe the implications of corn earworm resistance for resistance monitoring, regulatory policies, further research, and the sustainability of the pyramided Bt technology.

Dively *et al.*<sup>3</sup> measured changes in field efficacy and Cry toxin susceptibility to Maryland populations of *H. zea* using 21 years of field monitoring data from sweet corn hybrids expressing Cry1Ab (1996–2016) and Cry1A.105 + Cry2Ab2 (2010–2016) proteins. They demonstrate that since commercial introduction of these hybrids, larvae successfully damaged an increasing proportion of ears, consumed more kernel area, and reached later developmental stages (4<sup>th</sup> - 6<sup>th</sup> instars). These damages were not due to changes in population densities of corn earworm, which declined over past two decades as observed from light traps. Also, there was no difference among the commercially available Bt varieties expressing Cry1Ab, so the increasing damage was not due to a variety effect. Moreover, fitness of the Maryland *H. zea* strain was significantly lower than that of the susceptible strain as indicated by lower hatch rate, longer time to adult eclosion, lower pupal weight, and reduced survival to adulthood. Taken together, field

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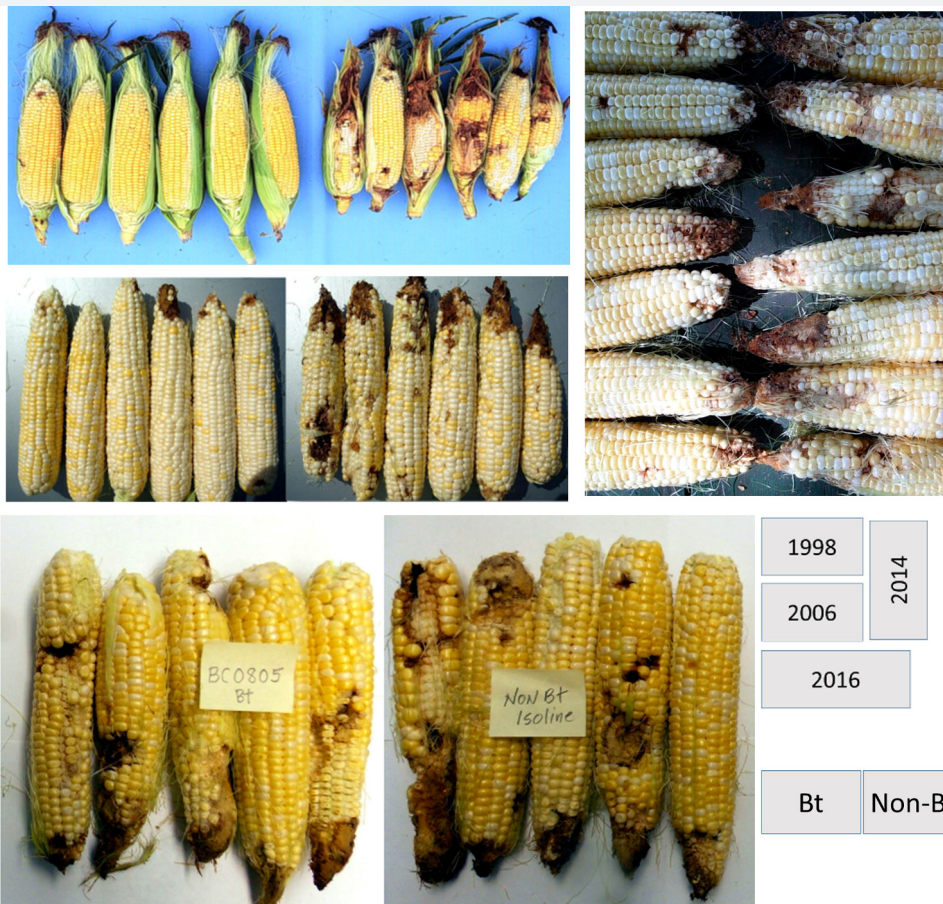
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efficacy comparisons and lab bioassays demonstrate strong evidence of field-evolved resistance in *H. zea* populations to multiple Cry toxins. The use of Bt crops has broadly helped reduce insecticide usage in the United States. However, many sweet corn farmers in Maryland either have stopped growing Cry1Ab / Cry1A.105 + Cry2Ab2 hybrids or are applying more insecticide sprays to compensate for the reduced control efficacy.



Trends of typical ear damage of BC0805 (Bt) expressing Cry1Ab untreated Providence (nonBt) and in Maryland 1998-2016.

**Factors contributing to resistance evolution**

The field-evolved resistance could be attributed to a range of factors, including corn earworm behavior and life history characteristics, Bt toxicity, trends in Bt acreage, and production practices. The high adoption rate of Bt field corn and cotton, along with the moderate dose expression of Cry1Ab and related Cry toxins in these crops and decreasing refuge compliance, probably contributed significantly to evolution of resistance. Regional deployment levels of Bt corn hybrids can also contribute to increasing resistant gene frequencies, with low technology adoption related to slower rate of resistance evolution<sup>5</sup>. The adoption rate of Bt field corn in Maryland is very high, accounting for 83–93% of the hectares planted during 2013 in crop reporting districts where the study was conducted. The moderate dose expression could allow heterozygous

resistant individuals containing minor resistance alleles to survive, thus increasing the frequency of genes that confer resistance in the *H. zea* population<sup>6</sup>. This high adoption of Bt corn, along with a decline in compliance with the refuge requirements, has almost certainly contributed at the local level to the evolution of resistance. Surveys show that 22% of the farmers are not complying with refuge requirements<sup>7</sup>.

The behavior and life history characteristics of *H. zea* may also be attributed to evolution of resistance. Successful overwintering of *H. zea* during most years in Maryland enables resistant individuals to contribute resistant alleles to the population in the subsequent year. The southerly flow weather patterns can facilitate northward dispersal of moths<sup>8</sup>. Moths already selected for resistance on Bt corn and cotton grown in southern U.S. can enhance resistant development in northern corn growing regions through this south-north migration. Also, behavioral changes in *H. zea* due to feeding on Bt corn (asynchrony of mating due to delayed development and emergence and alteration in cannibalistic behavior) may have increased the selective differential between susceptible individuals and those carrying resistance gene<sup>9</sup>.

### Implications

*H. zea* resistance to the Cry toxins is likely to increase and spread, with significant implications for resistance monitoring and management, regulatory policies, and sustainability of the Bt transgenic technology. An insect resistance management (IRM) plan aimed at delaying the onset of resistance is required by the U. S. Environmental Protection Agency (EPA) for commercial registration of Bt crops in the U. S.<sup>10</sup>. For IRM, deploying a high toxin expression to kill nearly all heterozygous resistant individuals in conjunction with non-Bt crops grown in proximity or amidst Bt crops as refuges is key. This 'high dose-refuge' strategy keeps the resistant allele frequency low by allowing survival and mating of susceptible insects with Bt resistant insects<sup>11</sup>. EPA mandates a 20% and 50% structured refuge in the corn belt and cotton growing regions, respectively, for Bt corn hybrids expressing single Cry protein. For corn hybrids expressing multiple pyramided Bt proteins, the EPA approved the refuge-in-the-bag (RIB) strategy with reduced refuge

requirement (95 % Bt : 5 % Non-Bt) in the corn belt<sup>10</sup>.

The RIB strategy primarily aims to manage evolution of resistance in European corn borer, *Ostrinia nubilalis* (Hübner), but not corn earworm. Hence, there still is a 20% structured refuge requirement for Bt corn with no RIB option in the southern U.S., which faces high *H. zea* population pressure<sup>4</sup> and additional selection pressure from Bt cotton. However, with Maryland *H. zea* populations now resistant to multiple Cry proteins, resistance is likely to increase and spread with the shift to RIB corn hybrids. Similarly, due to northward influxes of potentially resistant moths from southern source regions, the risk of further evolution of resistance may increase if refuge size is reduced (from 50% to 20%) in regions where Bt cotton is used. This is of relevance given the increasing damage and evidence of *H. zea* developing resistance to the Cry1Ab trait in field corn in the South and North Carolinas<sup>12</sup>.

The RIB strategy has also raised concerns about accelerated resistance evolution, apart from reductions in the refuge size, due to pollen-mediated gene flow between Bt and refuge plants in a seed blend. Cross-pollination between refuge and neighboring Bt plants or intermediate levels of toxin expressed in kernels can result in fewer susceptible *H. zea* moths, thereby diluting or nullifying the purpose of refuge<sup>13</sup>. Cross-resistance between Cry1Ab and other Cry toxins may further exacerbate resistance development to new pyramided traits in Bt field and sweet corn<sup>14</sup>.

Registrants of Bt corn are also required by the EPA to annually monitor potential changes in susceptibility of target insect species to Bt toxins in order to detect the evolution of resistance before field efficacy fails<sup>15</sup>. To date, however, corn earworm sampling for monitoring and testing for resistance have focused on the southern U.S. regions where both Bt corn and cotton are grown. Resistance in Maryland *H. zea* populations to multiple Cry proteins warrants expanded resistance monitoring for all Cry proteins registered against corn earworm and at all high corn production regions including the corn belt. Historically, Maryland represented the northern range for overwintering *H. zea*<sup>16</sup>. With these populations evolving resistance, programs monitoring for resistance in further northern ranges of *H. zea* are essential. Warming temperatures exacerbate the risk

of resistance spreading, as latitudes north of 40° become conducive for successful overwintering and increasing migration of *H. zea*<sup>17</sup>.

Venette *et al.*<sup>18</sup> proposed using sentinel Bt sweet corn as an in-field screen to monitor the evolution of target pest resistance and overall efficacy of Bt corn, and Dively *et al.*<sup>3</sup> demonstrate its applicability for quantifying control efficacy and resistance monitoring. Sweet corn as a sentinel host crop is a better diagnostic tool for monitoring early stages of resistance than field corn. Late season plantings of sweet corn are highly attractive to *H. zea* during the silking and ear development period. Importantly, infestation in sweet corn is higher than in field corn, which reduces the sample size of ears required to statistically detect changes in *H. zea* damage and susceptibility. Unlike field corn, sweet corn is harvested at a premature stage, thus toxin expression is consistently high throughout the crop cycle and generally higher in silk and kernel tissues than in Bt field corn. With the exception of Cry1F, the Bt events and Cry proteins expressed in field corn are also expressed in sweet corn, e.g., Attribute (expressing Cry1Ab toxin, event Bt11) and Attribute II (expressing Cry1Ab and Vip3A, event MIR162) hybrids from Syngenta Seeds, and Performance

Series (PS) hybrids (expressing the Cry1A.105 and Cry2Ab2 toxins, event MON89034) from Seminis Seeds. Finally, it is relatively easy to quantify changes in the incidence and severity of ear damage in sweet corn as a measure of control efficacy. Due to these factors, we recommend monitoring programs use sweet corn as a sentinel host crop.

The field-evolved resistance to multiple Cry toxins in *H. zea* also highlights some research needs. The mechanism through which corn earworm develop resistance to the pyramided traits needs detailed assessment. The activity and expression of alkaline phosphatase in the midgut binding sites is associated with *H. zea* resistance to Cry1Ac protein<sup>19</sup>. The role of alkaline phosphatase and other cellular mechanisms of resistance development to pyramided Bt varieties needs further investigation. Similarly, large scale population genetics and stoichiometric/stable isotopic analyses would help understand the role of migratory populations and landscape in evolution and spread of resistance. Finally, the influence of increased Bt adoption and climate change (temperature anomaly) on resistance evolution needs investigation. This is relevant given the predicted increase of overwintering range and climatic suitability for many of insect pests targeted by Bt crops.

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## CRISPR/Cas9 for Excising Chromosomal Fragments: A Viable Tool for Eliminating Marker Genes from Transgenic Plants

Vibha Srivastava

Biologists have long dreamed of devising practical methods of targeted genomic modifications. This dream has largely been realized upon the development of the Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) system (CRISPR/Cas9) as the gene-editing tool. Genomic modifications can generate phenotypic variation and provide new sources of genes for breeding crops. Of the gene editing techniques, CRISPR/Cas9 is quickly expanding, as it is the easiest, most effective, and most efficient system for genome targeting in many organisms, including plants<sup>1-3</sup>, and has been greatly simplified to a single module of Cas9 coupled to a single guide RNA (Cas9:sgRNA) as the gene-editing reagent<sup>4-6</sup>. The

fundamental basis of gene editing by Cas9:sgRNA lies in the ability of Cas9 in creating double-stranded breaks (DSB) at the chromosomal sites determined by the sgRNA. The breaks can be repaired by a host of cellular mechanisms, leading to deletion, insertion and/or replacement of the sequences. The most intuitive process of repairing DSBs is joining the two broken ends through non-homologous end joining (NHEJ). The NHEJ process, however, could result in insertions and/or deletions (indels) at the site, which serves well for targeted knock-outs by CRISPR/Cas9. Other DNA repair processes – base substitution or DNA replacement – are more sophisticated, and occur rarely in the mitotically dividing cells<sup>7,8</sup>. To address these challenges, biologists are devising methods to

harness homology-directed recombination (HDR), which will vastly expand the suite of gene editing applications. Therefore, while some facets of gene editing are technologically challenging, creating DSBs by CRISPR/Cas9 is highly efficient.

A major goal of crop breeding is to develop resilient plants that can withstand environmental threats and produce abundant food. The challenge is, however, that stress tolerance traits are generally polygenic, and a combination of genes will have to be introduced to develop resilient varieties. Genetic engineering is arguably the most effective approach for introducing polygenic traits, as it can stack the genes in a single locus, while conventional plant breeding affects assortments of alleles and linkages, many with undesirable traits. The latter is a major issue when non-crop varieties serve as the donor, *e.g.*, in ‘rewilding’ or reverse breeding<sup>9</sup> that is gaining attention, as stress tolerance traits are abundantly found in wild and extant varieties.

Genetic engineering, whether practiced conventionally or through gene editing approaches, involves plant cell transformation, selection, and regeneration of transgenic plants. Conventional genetic engineering based on random gene integration is a well-established technique in most crops, although HDR-mediated transformation methods are still evolving. Both approaches involve selection of the transformed events through tissue culture, and therefore, are reliant on selection marker genes (SMGs). The removal of SMGs from transgenic plants is an important part of the process and one that is encouraged by scientific panels and agencies regulating genetically engineered crops<sup>10,11</sup>.

### Excising Marker Genes from Transgenic Plants

For many reasons, scientific or technical, the persistence of SMGs in transgenic plants is considered risky to the environment, and certainly unnecessary for the plant. When antibiotic resistance genes serve as SMGs, the risks are potentially harmful, as gene escape could lead to a scenario of diminished antibiotics effect in human or livestock<sup>11,12</sup>. These risks can be avoided. Methods for removing SMG have been described for many years. These methods require incorporating specific designs or strategies

in the transformation vectors for performing SMG excision or segregation in the resulting transgenic plants or their progeny.

The most popular method utilizes *Cre-lox* site-specific recombination. In this method, a SMG is placed between *lox* sites for excision by the *Cre-lox* recombination<sup>13,14</sup>. The advantages of *Cre-lox* system include its robust activity and minimal off-target effects in most plants. However, it poses similar technical problems to complex genetic engineering in that it involves additional rounds of transformation and SMG excision. *Cre-lox* recombination leaves a reactive footprint (*lox* site) at the site of excision, which could recombine with *lox* sites introduced in subsequent transformations<sup>15,16</sup>. Therefore, alternative approaches for removing SMG will be useful for offering more flexibility in genetic engineering.

CRISPR/Cas9 has mostly been used for targeted mutagenesis at one or more sites leading to point mutations. Even in multiplexing with CRISPR/Cas9, the goal has been to create point mutations at multiple sites simultaneously. So far, little attention is given to fragment deletions that could occur if two sites are on the same chromosome or if a single gene is targeted by two sgRNAs. Indeed such deletions have been observed<sup>17-20</sup>, but a detailed characterization and heritability of the excision site is necessary for developing a viable marker-excision technology.

### Using CRISPR/Cas9 for Marker Excision

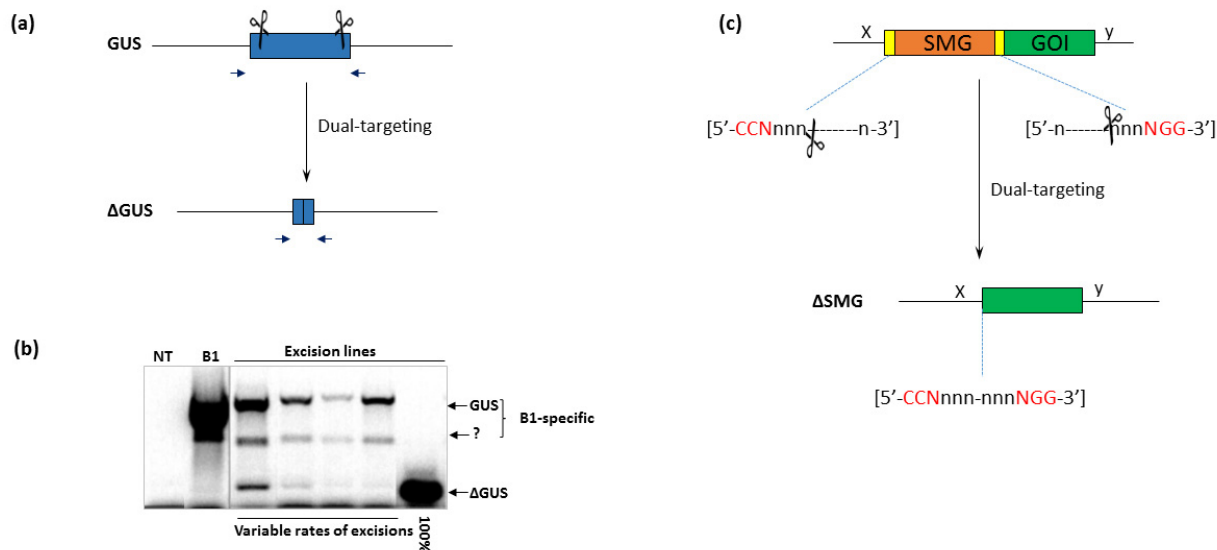
To evaluate CRISPR/Cas9 efficiency in excising marker genes, we targeted the  $\beta$ -glucuronidase (GUS) gene in the rice (*Oryza sativa* L.) genome cv. Nipponbare at two points (dual-targeting) with the goal to delete it upon simultaneous targeting<sup>21</sup> (Fig. 1a). We delivered the Cas9:sgRNA vector by either gene gun or *Agrobacterium* and collected >100 transformed (callus) events. We observed a low rate of dual-targeting in callus, as indicated by PCR assay, detecting GUS excision ( $\Delta$ GUS) in only ~5% of the events. Most interestingly, one-third of these events displayed 100% or bi-allelic excisions. Bi-allelic events are important in generating marker-free plants in the first (T<sub>0</sub>) generation. It is well-known that Cas9:sgRNA targeting occurs more efficiently during plant regeneration<sup>22</sup>. Indeed, we observed a dramatic

increase in excision rate in the regenerated transgenic plants, as almost all plants showed excision at variable rate (**Fig. 1b**). About 10% of the regenerated plants showed bi-allelic excision characterized by the presence of the excision site ( $\Delta$ GUS locus) and the absence of the intact site (GUS). The bi-allelic excision lines were further verified to contain 100% excision by Southern hybridization.

Since the goal of the marker-excision process is to generate stable marker-free plants, bi-allelic plants are the most desirable products. In addition, these plants will predictably transmit marker-free locus to the progeny. Although mono-allelic excisions will also be useful, it should be noted that mono-allelic excisions are difficult to distinguish from chimeric plants that might contain patchy/variable excisions in the tissue. Therefore, we used a more rigorous criterion of bi-allelic or 100% excisions for estimating

CRISPR/Cas9 efficiency in marker-excision. Using this criterion, we found the efficiency to be ~3% of all transgenic events (callus) and 10% of all regenerated plants. However, other lines showing excisions might also lead to marker-free plants in T1 or subsequent generations. Further, since progeny analysis will be an important part of the process to segregate out Cas9:sgRNA transgene, additional opportunities to identify marker-free plants will be available in case bi-allelic plants are not recovered.

In a practical sense, the estimated efficiency of CRISPR/Cas9 in excising marker genes should not pose a bottleneck in 'easy-to-transform' plant species such as rice. However, in 'difficult-to-transform' species, T1 and T2 progeny analysis might become an essential part of the process, requiring more effort in generating marker-free plants. In comparison, the Cre-lox system is reportedly more efficient in excising



**Figure 1: Dual-targeting by CRISPR/Cas9 for removing marker genes from transgenic plants.** (a) A locus containing GUS gene is targeted at both ends by Cas9:sgRNA (scissors) to delete GUS gene ( $\Delta$ GUS). (b) PCR using primers, shown as blue arrows in (a), clearly detects the excisions. A representative PCR shows detection of unexcised (GUS) or excised ( $\Delta$ GUS) sites. NT: non-transgenic and B1: parental plant. The B1 site also produces an unexpected (?) shorter amplicons, while excision is indicated by the presence of the  $\Delta$ GUS amplicon. The relative abundance of  $\Delta$ GUS amplicon was used to determine % excision. (c) To excise out the full-length of the selection marker gene (SMG) that is adjoining to the genes-of-interest (GOI) at the locus between x and y chromosomal markers, flanking target sites (yellow boxes) consisting of sgRNA complementarity and NGG PAM (red font) can be placed for dual-targeting by Cas9:sgRNA. The resulting  $\Delta$ SMG site is expected to contain precise excision of SMG and contain a predictable repair sequence (shown in parenthesis).

marker genes in diverse plant species<sup>14</sup>. Therefore, in simpler genetic engineering approaches, Cre-lox is a good, practical tool for removing marker genes. However, in performing complex genetic engineering, Cas9:sgRNA might be a better choice as it will allow downstream modifications without requiring strategies to circumvent cross-interaction with the previously inserted transgene sites.

### Precise Excision

Targeted mutagenesis by site-specific nucleases such as Cas9:sgRNA is based on the induction of DSB, which is mostly repaired by the error-prone NHEJ process. However, the goal of marker-excision approaches is to make an exact predictable change through precise excision of SMG. Cas9:sgRNA creates a blunt cut at 3-bp upstream of the protospacer-adjacent motif (PAM) sequence (NGG) at the target<sup>5</sup>. We sequenced the excision site ( $\Delta$ GUS) in more than 12 T0 plants, including the biallelic excision plants, and found that all contained a precise excision locus derived from the ligation of the two blunt ends produced by a cut exactly 3-bp upstream of the PAM sequences<sup>21</sup>. Further, T1 plants (13 analyzed), derived from one of the bi-allelic  $\Delta$ GUS plant, inherited the precise  $\Delta$ GUS locus and showed segregation of Cas9:sgRNA transgene (unpublished data).

This highly relevant feature of chromosomal excisions through dual-targeting has also been reported by others<sup>23,24</sup>. Hence, dual-targeting by Cas9:sgRNA is effective in precisely removing marker genes from transgenic plants. A more important issue to consider is that if SMG adjoins to the gene-of-interest without any buffer sequences, then sequences within SMG will have to be targeted. This would essentially mean that remnants of SMG will be found at the  $\Delta$ SMG locus, and which stretches of remnants will depend on how interior the PAM sites were located from the 5' and 3' ends of the SMG. This problem could be solved by adding 'target sites' for dual-targeting by a single or dual sgRNA (**Fig. 1c**), and designing the target sites rigorously to minimize off-target effects. Dual-targeting on identical sites might also lead to improved excision efficiency as opposed to targeting at the two dissimilar sites, where targeting efficiency might also differ.

### Not Just Marker Excision

CRISPR/Cas9-mediated deletion of chromosomal fragments could be applied for generating divergent gene functions, *e.g.*, by targeting 5' upstream region, where *cis*-regulatory elements are located. The *cis*-elements are important to developmental and environmental responses, and mutations in these elements could lead to phenotypic diversification. Many studies indicate that loss of *cis*-elements in the duplicated genes can lead to gain-of-function<sup>25-28</sup> through the evolutionary processes called sub- and neo-functionalization<sup>29,30</sup>. This evolutionary mechanism can be harnessed by targeting CRISPR/Cas9 to the 5' upstream regions of the genes.

In *Arabidopsis*, the genes rich in *cis*-elements are more likely to generate new functions upon elimination of one or a few *cis*-elements<sup>25</sup>. Many stress-response genes contain multiple *cis*-elements as they respond to a myriad to external stimuli, and therefore are good candidates for developing new genes of divergent functions. CRISPR/Cas9 will be effective in creating short deletions in the 5' upstream sequences for altering gene function and generating potentially new useful alleles. The application of CRISPR/Cas9 in driving neo-functionalization of genes could greatly enrich the gene pool for breeding improved crops.

### Conclusions

The CRISPR/Cas9 gene editing technology is highly efficient in creating DSBs through single- or multi-site(s) targeting. We have shown that dual-targeting by CRISPR/Cas9 in rice leads to precise excision of a marker gene, which can be easily detected by PCR<sup>21</sup>. About 10% of the plants representing 3% of transgenic events show bi-allelic or 100% excision, a trait that is transmitted to the next generation. Thus, CRISPR/Cas9 is useful for removing selection marker genes and developing marker-free transgenic lines.

Our work also implies that this technique will be useful for driving neofunctionalization of genes through targeted deletions of *cis*-regulatory elements of the genes. A deeper understanding of the expression patterns and gene functions of the duplicated genes will be extremely helpful in designing such deletions

in plant varieties suitable for breeding. However, in extending these applications to field grown crops, a thorough investigation of the edited genomes will be important, as risks associated with the inopportune release of deleterious mutations into crop varieties are not worth accepting.

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## Second Generation Maize Hybridization Technology Has Potential to Improve Food Security and Agricultural Sustainability

*Jim Gaffney and Bo Shen*

### **Nitrogen, heterosis, and hybrids**

Nitrogen is often a limiting factor of maximum yield potential in maize production. It is also one of the most expensive inputs for farmers. Plant available nitrogen is not stored in the soil and may be lost to ground and surface water or as nitrous oxide gas to the atmosphere<sup>1,2,3</sup>. Significant improvement in nitrogen use efficiency in North America has been realized through a combination of plant breeding, improved agronomics, and soil fertility management. Modern hybrids are more efficient at utilizing nitrogen<sup>4</sup>, and nitrogen fertilizer application rates have remained approximately flat since the 1980s, while maize yield has increased significantly<sup>5</sup>. In developing countries, a combination of highly weathered tropical soils with low organic matter, low nitrogen, and use of older open-pollinated varieties and poor quality hybrids severely limit maize productivity for smallholder farmers. Improvements in nitrogen use efficiency and hybrid seed systems will reduce further the environmental footprint of maize production while enabling continual productivity gains, especially for farmers in tropical countries.

Heterosis, often referred to as hybrid vigor, has been the basis of genetic gain in temperate maize for 90 years. Hybrid vigor is defined as an increase in the size or rate of growth of offspring when two unrelated parents are crossed<sup>6</sup>. Modern breeding programs have developed specific arrays of inbreds with good combining ability which are crossed to produce a hybrid. Only the best inbreds and hybrids are advanced from each breeding cycle after careful evaluation in the targeted environment. The continual breeding, evaluation, and rapid turnover of new hybrids help ensure that commercial hybrids are productive under current environmental conditions<sup>7</sup>.

Eliminating self-pollination and achieving cross-pollination of the female inbred, essential to creating a hybrid, has been accomplished in a number of ways over the years. Mechanical detasseling of the female inbred has been widely used and remains the main sterilization tool in many parts of the world, yet it is labor-intensive, time sensitive (it must be conducted before pollen is shed), and damages the upper leaves of the plant, often resulting in production of fewer hybrid seeds. Hybrid seed of rice, sorghum, and

maize also have been developed through the use of cytoplasmic male sterility (CMS), which is derived from mutations in mitochondrial DNA followed by restoration of male fertility with nuclear restorer genes. Drawbacks of CMS include the following: the complexity of developing a male-sterile line, a maintainer line, and a fertility restoration line; fertility and sterility are not always stable in all environments; and CMS does not work with all backgrounds, thus eliminating potentially valuable germplasm from a breeding program<sup>8,9</sup>.

Nuclear genetic male sterility is a strategy that offers an alternative to CMS. It is stable across germplasms and environments<sup>10</sup>, but its use in a hybrid system is challenging because the male-sterile female inbred cannot be self-pollinated. This obstacle was overcome by use of a hybrid seed production technology (SPT) construct containing an *Ms45* gene for fertility restoration, an  $\alpha$ -amylase gene for pollen disruption, and a seed color marker to allow seed sorting<sup>11</sup>. The maintainer line with this construct was fertile, with 50% of the pollen being transgenic *ms45* and 50% non-transgenic *ms45*. The transgenic pollen does not pollinate due to the presence of the  $\alpha$ -amylase gene, while the other 50% of the pollen is available to fertilize homozygous mutant plants. The progeny is homozygous *ms45*, a nontransgenic seed used for hybrid seed production. *Ms44* is a dominant male-sterile mutant that can be used to produce male-sterile inbreds by eliminating pollen formation early in the maize plant reproductive stage.

With *Ms44*, we now have the ability to maintain sterility in the hybrid production field during the inbred increase (which helps ensure high quality hybrids). The *Ms44* system fits well within the three-way hybrid production system used by most small and medium size seed companies in the tropics, and because pollen formation is eliminated relatively early the plant life-cycle, we have observed a yield increase in both inbreds and hybrids.

#### **Molecular basis of *Ms44* dominant male sterility**

The basis for dominant male sterility at the molecular level was revealed by map-based cloning of the *Ms44* gene. *Ms44* is expressed specifically in the tapetal cell layer of the anther during meiosis through the

quartet and uninucleate stages of pollen development and encodes a member of nonspecific lipid transfer protein family. This family of protein plays important roles in lipid transfer, pathogen defense, stress tolerance, and anther development<sup>12</sup>. A single amino acid change from alanine to threonine at the predicted signal peptide cleavage site blocks the secretion of *Ms44* mutant protein from the tapetum to the locule and results in the disruption of pollen development and dominant male sterility in maize. We have also demonstrated that *Ms44* protein itself is not critical for pollen development. Rather, tapetum secretion is likely a critical component for the dominant male sterile phenotype<sup>13</sup>.

#### **Nitrogen partitioning and use in *Ms44* plants**

The developing tassel and ear compete for nutrients starting early in the reproductive phase of the maize plant, so we hypothesized that *Ms44* dominant male sterility would impact this competition. Biomass and total N content of various plant parts were measured from reproductive stages V9 to V17 to evaluate the hypothesis. No differences between the *Ms44* and wild type plants in either biomass or total N were found in shoot tissues. Conversely, total N and biomass were reduced in the tassels of *Ms44* male sterile plants compared to the wild type. Ear biomass and total N increased in *Ms44* male sterile plants. By growth stage V17, tassel biomass was 64.7% less, ear biomass 24.6% greater, with more N was moving from the tassel to the ear, resulting in 9.6% more kernels in the *Ms44* sterile plants compared to the fertile wild type.

Higher grain yield in the field was also established by pollinating four elite *Ms44* male-sterile inbreds with four to five male testers and producing hybrid seeds for yield evaluation. Seed from wild-type female plants was 100% fertile (as expected) and was used as the control, while seed from heterozygous *ms44* female plants segregated 50% fertile and 50% male-sterile (the *Ms44* hybrid), also as expected. Multi-location trials were conducted under limited nitrogen conditions and optimal growing conditions. Across 17 hybrids evaluated, *Ms44* yield was 4% and 8.5% higher under low and ultra-low nitrogen conditions, respectively, compared to the control. Under optimal conditions, a small but significant increase (0.9%) in

yield was observed in the Ms44 hybrids. Importantly, we did not observe a yield penalty for Ms44 in any of the conditions evaluated<sup>13</sup>.

To ensure a viable breeding and seed system, the original SPT system<sup>11</sup> was modified to allow fertility restoration by silencing mutant Ms44 allele expression using an artificial miRNA in the maintainer construct, while the  $\alpha$ -amylase and color marker genes in the construct remain identical to those in the original SPT. Using this approach, silencing Ms44 expression in mutant plants restored fertility upon transformation of the construct into a heterozygous Ms44 inbred line. Transgenic pollen grains were nonviable due to the presence of  $\alpha$ -amylase expressed by a pollen-specific promoter, while seeds expressing red fluorescent protein were distinguishable from yellow, non-transgenic seeds under specific wavelength light.

The construct lines segregating independently for the native Ms44 locus were selected for advancement and T1 transgenic lines were self-pollinated to obtain Ms44 mutant and sibling maintainer lines. The Ms44 mutant lines had larger ears, more silks, and were shorter compared to fertile maintainer plants. Only non-transgenic, yellow seed progeny resulted from crossing the homozygous Ms44 male-sterile inbred with pollen from the maintainer, because transmission of the construct occurs only through female gametes. Pollen carrying the construct is nonviable due to the pollen-specific expression of  $\alpha$ -amylase.

Hybrid seed production is made practical with this system either when used to produce single-cross hybrids or 3-way hybrids because the female inbred is homozygous dominant for male-sterility. Therefore, in single-cross hybrid production, crossing with a male parent results in F1 hybrid seed which is also male-sterile. Blending of male sterile and male fertile hybrid to ensure pollination in a farmer's field is accomplished by adding a step after the initial maintainer line cross during inbred increase in which heterozygous Ms44 male-sterile female inbreds are generated by pollination of the male-sterile Ms44 homozygous inbred with the wild-type inbred. After crossing with the designated male parent for that hybrid, the result is 50% male-sterile hybrid progeny and 50% male-fertile progeny.

### Implications for Hybrid Production

Hybrid seed sold to a farmer requires the crossing of two inbred parents, which is accomplished by preventing pollen shed from the female inbred parent. The Ms44 technology, combined with the Seed Production Technology (SPT) process<sup>11</sup>, offers advantages over other systems from a number of perspectives. First, we demonstrated a yield advantage in number of seeds from Ms44 inbreds of over 9% relative to wild type, offering an important productivity improvement and a cost-of-goods decrease. Detasseling the female inbred, a labor-intensive, somewhat destructive, and often imperfect sterilization method, is no longer needed.

Second, Ms44 is a native genetic male-sterile mutant and is very stable across germplasm and environments. This is a distinct advantage over CMS, which is restricted to select germplasm or the Roundup hybridization system<sup>14</sup> that is dependent on herbicide applications that may be impacted by environmental conditions and requires precise timing over large areas.

Third, the progeny from the Ms44 system, either inbreds or commercial hybrid seed, is non-transgenic, which may be important for countries with challenging regulations for genetically engineered crops. And fourth, dominant Ms44 offers a simple and effective system for producing high quality, 100% male-sterile female inbreds which would reduce costs and improve productivity of hybrid seed production. An additional benefit is the yield improvement due to a less competitive tassel relative to the developing ear, especially in low nitrogen conditions.

An especially interesting application of Ms44 may be in tropical germplasm and soils. For more than 50 years, breeders of temperate maize have been selecting for a compact tassel, while in Africa this has yet to occur, so portioning more energy from an otherwise large energy sink to the developing ear may provide more yield advantage than was identified in this study, which was conducted with temperate maize germplasm. Further, tropical soils are generally highly weathered, low in organic matter, and contain limited inherent nitrogen, with the challenge compounded by expensive nitrogen fertilizer for the average smallholder farmer in many tropical countries. The

opportunity for lower cost hybrids, which are more productive under low nitrogen conditions, may create opportunities for farmers most in need of improved inputs<sup>7</sup>. As van Ittersum *et al.* describe<sup>15</sup>, the demand for grain in sub-Saharan Africa will more than triple by 2050, and productive improvements and investments in agricultural research are critical to a largely agrarian populace. Modern maize hybrids demonstrate

improved physiological efficiency in use of nitrogen<sup>4</sup>, contributing to improved balance of N with maize productivity, and resulting in approximately the same N application rates per hectare for the past 30 years, while maize yields have continued to climb. The Ms44 system offers an incremental boost in this efficiency, with positive implications for both food security and environmental stewardship.

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