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Genetic Engineering of Maize (*Zea mays*) for High-Level Tolerance to Treatment with the Herbicide Dicamba†

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Herbicide-tolerant crops have been widely and rapidly adopted by farmers in several countries due to enhanced weed control, lower labor and production costs, increased environmental benefits, and gains in profitability. Soon to be introduced transgenic soybean and cotton varieties tolerant to treatments with the herbicide dicamba offer prospects for excellent broadleaf weed control in these broadleaf crops. Because monocots such as maize (*Zea mays*) can be treated with dicamba only during a limited window of crop development and because crop injury is sometimes observed when conditions are unfavorable, transgenic maize plants have been produced and tested for higher levels of tolerance to treatment with dicamba. Maize plants expressing the gene encoding dicamba monooxygenase (DMO) linked with an upstream chloroplast transit peptide (CTP) display greatly enhanced tolerance to dicamba applied either pre-emergence or postemergence. Comparisons of DMO coupled to CTPs derived from the Rubisco small subunit from either *Arabidopsis thaliana* or *Z. mays* showed that both allowed production of transgenic maize plants tolerant to treatment with levels of dicamba (i.e., 27 kg/ha) greatly exceeding the highest recommended rate of 0.56 kg/ha.

KEYWORDS: Dicamba; maize; transgenic; herbicide tolerance; corn

INTRODUCTION

The advent of herbicide-tolerant crops has provided agricultural producers with powerful new tools in the continuing quest to minimize production losses due to weed infestations of crops (*1*). Plant varieties tolerant to glyphosate, glufosinate, and other herbicides (*1*,*2*) have improved weed control, lowered production costs, made weed management practices easier, and contributed to reduced soil erosion due to more facile use of conservation tillage practices. The rapid and extensive adoption of herbicide-tolerant crops in the United States, Canada, Brazil, Argentina, and other countries (*1*) is a clear indication of the value these traits have brought to the agricultural economy.

To provide farmers with a broader choice of herbicide tolerance traits, we recently developed plants that are tolerant to the herbicide dicamba (*3*). Dicamba is a broadleaf herbicide that has been used to kill a broad spectrum of dicotyledonous weeds in corn and wheat crops since the 1960s. The isolation and genetic engineering of the dicamba monooxygenase gene from the dicamba-degrading bacterium, *Pseudomonas maltophilia*, strain DI-6, and its expression in broadleaf plants provided strong protection against treatments with levels of dicamba several-fold higher than the highest levels of dicamba recommended for most crops with which it is used (i.e., 0.56 kg/ha) (*3*). The dicamba tolerance gene has been incorporated into soybean and cotton varieties that are in late stages of development prior to entering the marketplace.

The availability of dicamba-tolerant soybean and cotton varieties will provide farmers not only a new means of controlling broadleaf weeds in broadleaf crops but also a significant tool for combating present herbicide-resistant weeds and stemming the appearance of new types of herbicide-resistant weeds (*3*,*4*). Likewise, stacking of the dicamba tolerance trait with other herbicide tolerance traits will allow farmers to use rotations of herbicides or combinations of herbicides in fashions that will contribute to suppressing the evolution of weeds resistant to any of the herbicides used in rotation or combination.

The dicamba tolerance gene encodes the enzyme dicamba monooxygenase (DMO) (*5*), from the bacterium *Pseudomonas maltophilia*, strain DI-6 (*6*). This enzyme inactivates dicamba by removal of an *O*-methyl group from the aromatic ring of the herbicide. To do so requires the presence of two other enzymes, a reductase and a ferredoxin, and a reducing source such as NADH (*7*). Earlier studies demonstrated that if DMO was targeted to the chloroplasts of transgenic broadleaf plants such as *Arabidopsis*, tobacco, soybean, and tomato, the presence and expression of the other two bacterial enzyme components were not required for dicamba inactivation by DMO, likely due to the

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availability of reduced chloroplast ferredoxin in place of the structurally similar ferredoxin from *P. maltophilia*.

Although dicamba has been used for over 50 years for the effective control of most broadleaf weeds in maize, some crop damage can occur if dicamba is applied to corn crops outside the developmental window prescribed on the package label, in certain years with atypical climactic conditions, in certain soil types, and/or with maize production on certain types of soil (*8*−*12*). Thus, development of maize that displays substantially enhanced tolerance to treatment with dicamba coupled with the ability to spray maize crops pre- or postemergence and under various climactic conditions may be appealing to farmers. Here we report the development of transgenic maize events expressing nuclear CTP-DMO genes that provide tolerance to treatments with high levels of dicamba applied either pre- or postemergence.

MATERIALS AND METHODS

Chemicals and Standard Methods. Chemicals for plant tissue culture were obtained from Fisher Scientific, Invitrogen, and Sigma. Restriction enzymes and other enzymes were purchased either from Fermentas or Invitrogen. A commercial formulation of dicamba (Clarity from BASF) used to test the tolerance of transgenic maize to dicamba was obtained from BASF.

Maize Transformation. *Construction of Expression Vector*. The native DMO *DdmC* (AY786443) from *P. maltopholia*, strain DI6 (*5*), and fusions of the DMO coding region with chloroplast transit peptide coding regions and other components were made in the cloning vector, pSK, and subsequently transferred to binary vectors (see below). Constructs containing (CaMV) 35S promoter (GenBank accession no. V00141 J02048), rice actin intron *OsAct1* (GenBank accession no. EU155408), and *Arabidopsis* heat shock protein gene 3′ termination region (GenBank accession no. BT006090) were obtained from Dr. Paul Feng, Monsanto Co. (St. Louis, MO). The 35S-AtCTP-DMO construct contains the coding region for the chloroplast transit peptide from the *Arabidopsis* RBCS1A (ribulose bisphosphate carboxylase small subunit 1A) *ats1A* gene (ID 843029), whereas the 35S-DMO construct lacks such a chloroplast transit protein coding region upstream of the DMO gene. The 35S-ZmCTP-DMO construct contains the coding region for the chloroplast transit peptide of the ribulose bisphosphate carboxylase small subunit 1 *ssu1* (GenBank accession no. BT040081) from maize.

The respective DMO cassettes were cloned into *Agrobacterium* binary vector pPZP211 (*13*) for plant expression. The T-DNA region of pPZPZ211 contains a multiple cloning site for inserting genes of interest and a neomycin phosphotransferase (*npt*) plant selectable marker cassette under control of the 35S CaMV promoter. The DMO cassettes were subcloned into pPZP211 between the *Pst*I and *Bam*HI sites of the vector. The respective DMO gene cassettes in the binary vector, pPZPZ211, were mobilized into *Agrobacterium tumefaciens* strain C58C1/pMP90 (rif/gent) by triparental mating using an *Escherichia coli* strain carrying the pRK2013 helper plasmid (*14*).

Plant Transformation. Agrobacterium-mediated transformation of *Zea mays* line Hi II was conducted as previously described (*15*).

Treatments with Dicamba. For treatments with dicamba, plants at appropriate growth stage (see Results) were sprayed with Clarity (dicamba) by the weed science unit of the Department of Agronomy and Horticulture at the University of Nebraska, under greenhouse conditions, using a compressed air, motor-driven, track sprayer with a flat-fan 8002E nozzle traveling at 1.87 mph. The solution containing active ingredient at various concentrations was applied at 182 L/ha.

RESULTS

To determine if increased tolerance to treatments with dicamba could be achieved in maize, we transformed the *Z. mays* line Hi II (*16*) with various cassettes harboring the DMO gene, including the three constructs shown in **Figure 1**. These constructs all contain the 35S promoter from cauliflower mosaic virus (*17*) and the 3′ termination region from the *Arabidopsis thaliana* heat shock 17 gene (*18*). They differ in that the 35S-DMO construct

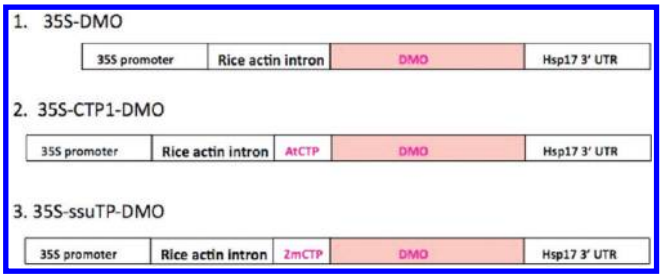

Figure 1. Gene constructs used in maize transformation: DMO gene cassettes without (1) and with coding regions for chloroplast transit peptides from the *Arabidopsis thaliana* Rubisco small subunit gene and the *Zea mays* Rubisco small subunit gene (2 and 3, respectively) used in the production of transgenic maize plants.

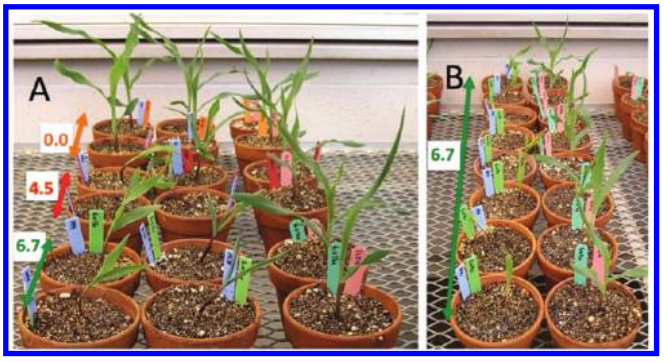

Figure 2. Comparison of damage due to treatment of transgenic and nontransgenic maize plants with dicamba applied pre-emergence: (**A**) herbicide symptoms of nontransgenic H 99 (left file), nontransgenic Hi II (middle file), and transgenic Hi II containing the 35S-ZmCTP-DMO gene (right file) sprayed with dicamba at 0 kg/ha (back two rows), 4.5 kg/ha (middle two rows), and 6.7 kg/ha (front two rows): (**B**) comparison of growth of nontransgenic Hi II plants (left file) and transgenic Hi II plants containing the 35S-ZmCTP-DMO gene both treated with dicamba at 6.7 kg/ha. Plants are pictured at 15 days after planting.

lacks a chloroplast transit protein (CTP) coding region upstream of the DMO gene, whereas the 35S-AtCTP-DMO construct contains the CTP coding region from the *Arabidopsis* Rubisco small subunit gene and 35S-ZmCTP-DMO contains the CTP coding region of the Rubisco small subunit gene from maize. With the construct containing no CTP, we produced 10 independent maize transformation events, with the construct containing the ZmCTP, we also produced 10 events, and with the construct containing the AtCTP, 5 events were produced. In addition to the 3 constructs reported here, 10 additional constructs containing the DMO gene driven by various promoters and with different intron components and transit peptide elements were used to produce transgenic maize plants. Results with these events were similar to those obtained with the three constructs presently described (data not shown).

Because maize generally has good tolerance to treatment with dicamba at the highest label-recommended rate (0.56 kg/ha or 0.5 lb/acre), clear symptoms of dicamba damage generally appear only at high rates of application (*10*, *12*). To differentiate true dicamba injury in the maize Hi II background, we performed our greenhouse-based tests for enhanced tolerance to dicamba applied postemergence using the extraordinarily high rates of 12, 24, 36, and 48 times the highest recommended rates (i.e., 6.75, 13.5, 20.25, and 27 kg/ha). When applied pre-emergence, dicamba damage is elicited at lower concentrations. This is illustrated in **Figure 2A**, which shows that transgenic Hi II maize events

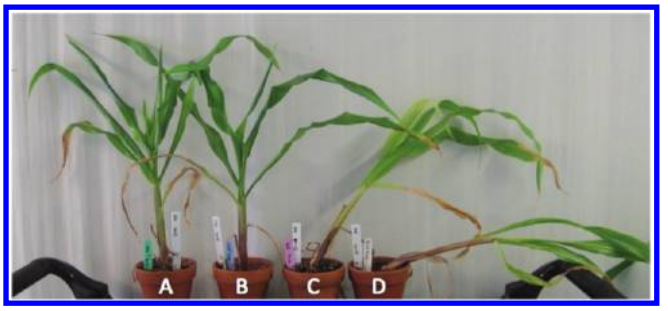

Figure 3. Comparisons of transgenic maize plants expressing DMO containing the *Arabidopsis* Rubisco small subunit gene transit peptide (**A**) or the maize Rubisco small subunit gene transit peptide (**B**) with nontransgenic lines B73 (**C**) and Hi II (**D**) 2 weeks after treatments with dicamba at 27 kg/ha.

expressing the 35S-ZmCTP-DMO gene construct are completely tolerant to dicamba applied pre-emergent at 6.7 kg/ha, whereas nontransgenic Hi II plants treated with identical amounts pre-emergent show distinct symptoms (e.g., stalk leaning and bending) at 4.5 and 6.7 kg/ha. Stunting of nontransgenic plants by pre-emergent application of dicamba at 6.7 kg/ha and lack of stunting and stalk leaning in transgenic maize plants planted and treated at the same times are illustrated in **Figure 2B**.

Figure 3 illustrates the strong tolerance of Hi II plants transformed with the 35S-AtCTP-DMO construct and Hi II plants transformed with the 35S-ZmCTP-DMO construct to postemergent treatments with dicamba at 27 kg/ha. Similarly treated nontransgenic B73 [parent used to create Hi II genotype (*16*)] and Hi II plants displayed stalk bending and leaning, a classic auxin response and the most conspicuous phenotype of maize plants treated with excessively high levels of dicamba (*10*, *12*). The reason for this phenotype is evident in the photographs in **Figure 4**, which compare the root systems of nontransgenic Hi II maize plants photographed 2 weeks after treatment of plants with 27, 6.7, and 0 kg/ha dicamba (**Figure 4A**, **B**, and **C**, respectively). In contrast, Hi II plants transformed with construct 35S-ZmCTP-DMO (**Figure 4D**) or 35S-AtCTP-DMO (**Figure 4E**) treated with dicamba at 6.7 kg/ha had root systems similar to those of non-treated Hi II plants (**Figure 4C**). Control Hi II nontransgenic progenitor plants displayed marked stunting of roots when treated with dicamba at 6.7 kg/ha (**Figure 4F**). The root system of dicamba-treated nontransgenic plants developed to a much lesser extent than the root system of the transgenic plants, especially near the crown of the plant that gives rise to prop roots.

Data shown in **Figure 5** provide evidence that to achieve high tolerance of transgenic maize plants to treatments with dicamba requires expression of DMO bearing an N-terminal CTP. This is illustrated by comparing two dicamba-treated transgenic Hi II plants of the same age, one expressing the chimeric 35S-ZmCTP-DMO gene construct (**Figure 5A1**) and the other transgenic Hi II plant expressing the 35S-DMO gene construct lacking a CTP sequence (**Figure 5B1**). Importantly, the plant expressing the 35S-ZmCTP-DMO gene was treated with 20 kg/ha, whereas the plant expressing the 35S-DMO gene was treated at a rate of only 6.7 kg/ha. Calibration of the marked difference in size of the two plants can be gauged by the length of the 40 cm paper bags. Examination of the crown areas of both plants (**Figure 5A2**,**B2**) likewise reveals a marked difference in prop root development. Measurements of DMO expression levels in each of the two plants are provided by the protein blot analysis depicted in **Figure 5C**. This analysis demonstrates that both transgenic plants are producing DMO at similar levels (Hi II 35S-ZmCTP-DMO plant extracts, lanes 3 and 6 marked with a "+" sign, and Hi II35S-DMO plant extracts,

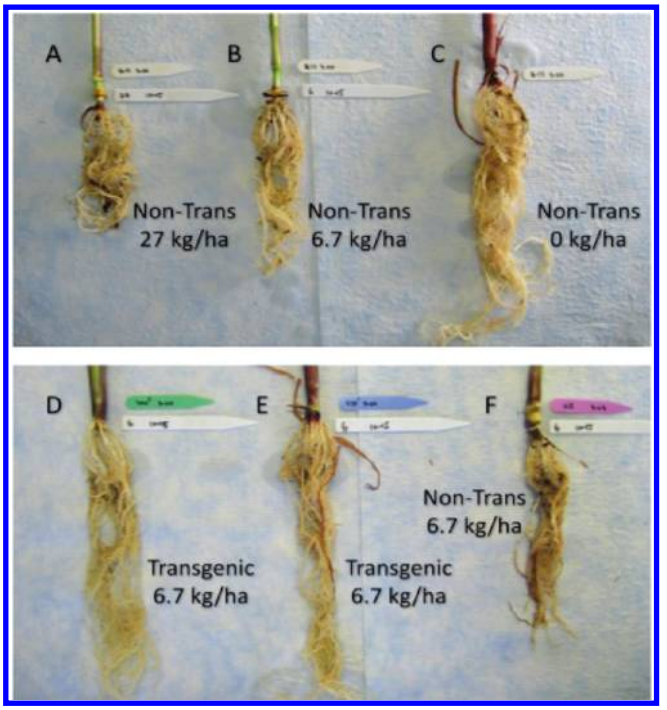

Figure 4. Comparison of root systems of Hi II nontransgenic maize and transgenic Hi II maize containing the DMO gene treated and nontreated with dicamba: (**A**) nontransgenic Hi II maize treated with 27 kg/ha dicamba; (**B**) nontransgenic Hi II maize treated with 6.7 kg/ha dicamba; (**C**) nontransgenic Hi II maize not treated with dicamba; (**D**) transgenic Hi II maize expressing the 35S-ZmCTP-DMO gene treated with 6.7 kg/ha dicamba; (**E**) transgenic Hi II maize expressing the 35S-AtCTP-DMO gene treated with 6.7 kg/ha dicamba; (**F**) nontransgenic Hi II maize treated with 6.7 kg/ha dicamba.

lane 11 marked with a "−" sign). This protein blot also illustrates that ZmCTP-DMO uptake and processing by the chloroplast are apparently complete (lanes 3 and 6), whereas uptake and procession of At-CTP DMO appear to be incomplete (lanes 4 and 7). Despite the incomplete processing, plants expressing the 35S-AtCTP-DMO gene displayed high-level tolerance to DMO treatments (data not shown).

DISCUSSION

Dicamba has been used in the past for control of broadleaf weeds in fields of corn and wheat because of the relative selectivity of dicamba in killing dicotyledonous plants at concentrations that usually have minimal effects on monocotyledonous plants. The development of maize with complete tolerance to levels of dicamba employed in agriculture eliminates uncertainties regarding climatic conditions and application times that might lead to crop damage (*8*−*12*). Our present studies with dicamba-tolerant maize indicate (as have studies with dicamba-tolerant broadleaf crops) that dicamba can be applied at recommended rates to DMO-containing crop plants either pre- or postemergence with no ill effects (**Figure 2**). Thus, dicamba-tolerant crops, in the future, may offer farmers the opportunity to use dicamba as a "burn down" herbicide, an option that may be particularly favorable in situations in which glyphosate-resistant broadleaf weeds are present in the area. In the near future, it appears likely that seed companies will stack two or more herbicide tolerance traits into individual crop varieties (e.g., cotton varieties triply stacked with glyphosate, glufosinate, and dicamba tolerance genes). This will allow the use of herbicide mixtures (e.g., dicamba and glyphosate) and herbicide rotations to control present herbicide-resistant

Figure 5. Requirement for a chimeric chloroplast transit peptide-DMO enzyme in transgenic maize to obtain high-level tolerance to dicamba treatment. Transgenic Hi II maize containing the 35S-ZmCTP-DMO gene (**A1**, **A2**) and transgenic Hi II maize containing the 35S-DMO gene lacking a chloroplast transit peptide coding region (**B1**, **B2**) were simultaneously treated postemergence at 15 days after planting with 20 kg/ha dicamba (**A1**, **A2**) or 6.7 kg/ha (**B1**, **B2**), respectively. Plants were photographed 75 days after planting to illustrate morphological and size differences (paper bag length = 40 cm) (**A1**, **B1**) and differences on prop root development (**A2**, **B2**). (**C**) Detection of DMO expression in transgenic and nontransgenic maize plants using protein blots incubated with anti-DMO antibodies. Lanes: 1, native DMO produced in *E. coli*; 2 and 5, protein extracts of transgenic Hi II maize not expressing DMO; 3 and 6, protein extracts of the transgenic Hi II maize shown in **A1** and **A2** expressing DMO from the 35S-ZmCTP-DMO gene (+ = plus CTP); 4 and 7, protein extracts of the transgenic Hi II maize expressing DMO from the 35S-AtCTP-DMO gene; 8, protein extracts of nontransgenic parental Hi II; 9, protein extracts of nontransgenic parental H99; 10, protein extracts from a transgenic Hi II maize transformant expressing DMO from the35S-ZmCTP-DMO gene; 11, protein extracts from a transgenic Hi II maize transformant expressing DMO from the 35S-DMO gene lacking a CTP coding region (− = minus CTP). 37 kDa = migration of size marker.

weeds, control the spread of these weeds, and slow the evolution of new types of herbicide-resistant weeds. Such an approach will be a powerful aid to farmers in maintaining the economic payoffs, ease of production practices, and environmental advantages they have come to depend on from the availability of herbicide-tolerant crops.

Periodic abnormal weather conditions, poorly timed applications of dicamba during maize development, or simple mistakes resulting in applications of higher than recommended concentrations of dicamba can lead to crop damage and financial losses (*8*, *10*−*12*). The high levels of dicamba tolerance demonstrated in greenhouse-grown maize herein likely minimize the chances of crop injury due to these situations. Although pre-emergent application of moderately high concentrations of dicamba to nontransgenic maize caused marked injuries (**Figure 2**), transgenic plants expressing DMO displayed good tolerance. Such levels of protection appear to be more than adequate to allow safe preplant or planting-time burn down of noxious broadleaf weeds, especially those resistant to treatment with glyphosate.

The first step in dicamba degradation in the bacterium *P. maltophilia*, strain DI-6, is the conversion of dicamba to 3,6-dichlorosalicylic acid (DCSA) by the three-component enzyme system, dicamba *O*-demethylase (*7*). The three components, a reductase, a ferredoxin, and an oxygenase (DMO), comprise a short electron transfer chain in which electrons from NADH are moved sequentially from the reductase to the ferredoxin and to the oxygenase, where they are used to "activate" an oxygen molecule for use in oxidation of the extracyclic methyl group to form formaldehyde and the herbicidally inactive compound DCSA. In plants it has been shown that all three bacterial components needed for dicamba inactivation in bacteria are not required, if DMO is targeted to plastids (*3*). The rationale was that the structure of the bacterial ferredoxin component of dicamba *O*-demethylase appeared to be quite similar to that of ferredoxin found in plant chloroplasts and, therefore, might substitute for the bacterial ferredoxin in shuttling electrons to DMO. Thus, in designing constructs for use in producing maize plants tolerant to dicamba treatment, inclusion of a CTP was not in question; the only question was if any of the presently available CTPs would prove to be adequate and, if so, would one prove better than the others? In the present studies, 96 transgenic maize events were generated and tested, some producing DMO preceded by the CTP from the *A. thaliana* Rubisco small subunit, some producing DMO with the *Z. mays* Rubisco small subunit, and others producing DMO lacking a CTP (**Figure 1**). Direct comparisons of these plants (**Figure 3**) and numerous other plants (data not shown) treated with extremely high dicamba concentrations (i.e., 27 kg/ha) demonstrated that both the *Arabidopsis* and *Z. mays* Rubisco small subunit CTPS were more than adequate when coupled to DMO to produce fully healthy dicamba-tolerant maize plants.

Figure 5 provides a direct comparison of maize plants expressing the DMO enzyme with an N-terminal CTP and treated postemergence with dicamba at a rate of 20 kg/ha (**Figure 5A1**) with a transgenic plant expressing the DMO enzyme lacking a CTP and treated with dicamba at only 6.7 kg/ha (**Figure 5B1**). These results illustrate the need for a chimeric CTP-DMO protein to provide high-level tolerance to dicamba treatments in transgenic maize. Although both transgenic plants produced mature DMO of the correct size and in similar quantities (**Figure 5C**), the presence of DMO outside the chloroplast (in the case of plants expressing the 35S-DMO gene) provides little or no protection against treatments of plants with dicamba. This observation is consistent with the hypothesis (*3*) that reduced ferredoxin in the chloroplast is needed in place of the bacterial reduced ferredoxin that drives DMO enzymatic activity in *P. maltophilia*, strain DI-6, from whence the DMO gene was isolated (*5*−*7*). Elite varieties of maize expressing DMO genes containing a CTP coding region and exhibiting strong dicamba tolerance have been produced and presently are in phase two (of four phases) of commercial development (http://monsanto.mediaroom.com/index.php?s=43&item=788).

The anticipated availability of dicamba-tolerant soybean and cotton plants in the near future will provide farmers with important new tools for efficient, cost-effective, and environmentally safe weed control. Importantly, these new tools also should prove highly valuable in the effort to control weeds that are resistant to glyphosate and other herbicides (*3*, *19*) by preventing the spread of these weeds and suppressing the appearance of new types of herbicide-resistant weeds. The evolution of herbicide-resistant weeds and their potential spread to additional prime agricultural areas is a growing treat to the continued use of herbicide-tolerant crops, crops that have allowed farmers in the Americas and elsewhere increased ease of crop production, good economic returns on investments in transgenic seeds, and better stewardship of erosion-prone soils (*1*). Development of maize and other crop plants tolerant to dicamba, and a range of other herbicides, allows herbicide combinations and rotation strategies that offer farmers a means to greatly prolong the effective lifetime of highly prized herbicide-tolerant crop technologies.

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