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Dominant mutations causing alterations in acetyl-coenzyme A carboxylase confer tolerance to cyclohexanedione and aryloxyphenoxypropionate herbicides in maize

(tissue culture selection/*Zea mays*)

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ABSTRACT **A partially dominant mutation exhibiting increased tolerance to cyclohexanedione and aryloxyphenoxypropionate herbicides was isolated by exposing susceptible maize (*Zea mays*) tissue cultures to increasingly inhibitory concentrations of sethoxydim (a cyclohexanedione). The selected tissue culture (S2) was >40-fold more tolerant to sethoxydim and 20-fold more tolerant to haloxyfop (an aryloxyphenoxypropionate) than the nonselected wild-type tissue culture. Regenerated S2 plants were heterozygous for the mutant allele and exhibited a high-level, but not complete, tolerance to both herbicides. Homozygous mutant families derived by self-pollinating the regenerated S2 plants exhibited no injury after treatment with 0.8 kg of sethoxydim per ha, which was >16-fold the rate lethal to wild-type plants. Acetyl-coenzyme A carboxylase (ACCase; EC 6.4.1.2) is the target enzyme of cyclohexanedione and aryloxyphenoxypropionate herbicides. ACCase activities of the nonselected wild-type and homozygous mutant seedlings were similar in the absence of herbicide. ACCase activity from homozygous tolerant plants required >100-fold more sethoxydim and 16-fold more haloxyfop for 50% inhibition than ACCase from wild-type plants. These results indicate that tolerance to sethoxydim and haloxyfop is controlled by a partially dominant nuclear mutation encoding a herbicide-insensitive alteration in maize ACCase.**

Acetyl-coenzyme A carboxylase [ACCase; acetyl-CoA: carbon-dioxide ligase (ADP-forming) EC 6.4.1.2] is the herbicide target enzyme in monocotyledonous species susceptible to the cyclohexanedione and aryloxyphenoxypropionate herbicides (1–6). ACCase catalyzes the ATP-dependent carboxylation of acetyl-CoA to form malonyl-CoA, the precursor for fatty acid synthesis. Herbicide-mediated inhibition of ACCase is thought to result in depletion of cellular malonyl-CoA, thus accounting for inhibition of acetate incorporation into free fatty acids in herbicide-treated maize chloroplasts (7) and into total lipids in maize root tips (8). The role of ACCase in determining plant responses to cyclohexanediones and aryloxyphenoxypropionates has been demonstrated in comparisons between tolerant dicotyledonous species and susceptible grass species (1–6) and between tolerant and susceptible species within the grass genus *Festuca*. For example, red fescue (*F. rubra*) is herbicide-tolerant and exhibits ACCase activity that is substantially less sensitive to inhibition by sethoxydim, a cyclohexanedione, and haloxyfop, an aryloxyphenoxypropionate, compared with susceptible tall fescue (*F. arundinacea*) (9). Alterations of ACCase that confer herbicide tolerance within a single grass species, including maize, have not been reported.

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Maize genotypes with tolerance to weed-controlling rates of cyclohexanedione and/or aryloxyphenoxypropionate herbicides would allow selective postemergence control of grass weeds in maize. This practice has the potential to reduce the amount of herbicides applied to maize. Preemergence herbicide treatments could be reduced with greater reliance on postemergence treatments that would only need to be applied if weed population density exceeded economic threshold levels. Furthermore, adopting a postemergence weed-control strategy based on sethoxydim-tolerant maize may give producers impetus to rely on cultivation-based weed-control programs. Sethoxydim could be used with herbicide-tolerant maize to control grass weeds that escaped cultivation-based weed-control programs.

We previously have shown that variation for tolerance to sethoxydim and haloxyfop can be identified in maize by using tissue culture selection of a nonregenerable 'Black Mexican Sweet' maize culture (10). ACCase in the variant cell lines was increased >2-fold compared to ACCase from unselected wild-type cultures and exhibited wild-type herbicide-sensitive inhibition kinetics. Increased ACCase activity in the tolerant cell lines was due to overproduction of the enzyme (10). We report here the characterization of a sethoxydim-tolerant mutant selected from a regenerable maize tissue culture. Tolerance was expressed in regenerated plants and their progeny, demonstrating that a heritable mutation conferred herbicide tolerance. Herbicide tolerance in the tissue culture, regenerated plants, and certain progeny was associated with an altered form of ACCase that was less sensitive to herbicide inhibition.

MATERIALS AND METHODS

Maize Tissue Cultures and Media. Friable, embryogenic callus cultures were established by plating immature embryos from the cross A188 × B73 onto solid N6 medium (11) containing 2% sucrose, 25 mM L-proline, and 100 mg of Casamino acids and 1 mg of 2,4-dichlorophenoxyacetic acid per liter. Callus maintenance and plant regeneration were conducted according to Armstrong and Green (12). Regenerated plants isolated from the callus surface were transferred to medium containing no hormones to allow further root and shoot development. Plants at the two- to three-leaf stage were transplanted to peat pots containing potting soil and vermiculite (1:1, vol/vol) and were grown in a culture room for 7–14

Abbreviations: ACCase, acetyl-CoA carboxylase; DTT, dithiothreitol.

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days. Plants were transferred to soil in 10-cm-diameter pots and either grown in the greenhouse or transplanted into the field.

Selection for Herbicide Tolerance. Approximately 100 g of callus was transferred to N6 medium containing 0.5 μM sethoxydim. Technical-grade sethoxydim (BASF) was diluted in 95% ethanol, filter-sterilized, and added to cool, sterile N6 medium. Sethoxydim-containing medium was used immediately or stored in low light. Each Petri plate contained five 0.5-g callus pieces. After 2 weeks of growth, each individual callus piece was subcultured onto one Petri plate and was maintained as a callus line during subsequent selection. At 2-week intervals the most vigorously growing 0.5 g of tissue from each callus line was subcultured into fresh medium containing increased sethoxydim concentration (0.5, 1.0, 2.0, 5.0, and 10.0 μM). A callus line that grew on 10 μM sethoxydim was presumed to be herbicide-tolerant and was designated line S2. Nonselected wild-type callus from the original source tissue culture was maintained on medium without herbicide.

Herbicide Tolerance. Callus (≈20 g fresh weight) of the nonselected line and S2 was grown in the absence of herbicide for 2 weeks. Samples of callus (0.5 g) were evenly spread onto a sterile 7-cm Whatman no. 1 filter paper disk placed directly onto culture medium containing various sethoxydim or haloxyfop concentrations. Technical-grade haloxyfop (Dow Chemical) was added to N6 medium prior to autoclaving. Two experiments for each chemical with three replicates of each herbicide concentration were conducted. After 2 weeks of growth, dry weights were recorded for callus dried at 60°C for 48 hr. Dry-weight increases were determined and growth was expressed as a percent dry-weight increase of the untreated callus.

Plants regenerated from the tissue cultures and their progeny were treated with sethoxydim and haloxyfop concentrations determined from commercial formulations to be equivalent to field application rates. Crop oil concentrate (2.3 liters per ha) was used as a wetting agent. Herbicide injury was visually evaluated 14 days after treatment.

ACCase Activity. Callus was collected from one Petri plate of the nonselected and herbicide-tolerant S2 lines at 4, 7, and 11 days of a 2-week growth cycle and evaluated separately for ACCase activity. Callus (5 g) was homogenized in a 4°C mortar containing 10 ml of 0.1 M Tricine·KOH, pH 8.3/0.3 M glycerol/5 mM dithiothreitol (DTT). The homogenate was filtered through two layers of Miracloth and centrifuged at 30,000 × *g* for 30 min. Supernatant (2.5 ml) from each line was desalted on a Sephadex G-25 column (1.5 by 5 cm) equilibrated with 0.1 M Tricine·KOH, pH 8.3/0.3 M glycerol/2 mM DTT. The extract was used directly for enzyme assays. For ACCase activity determinations from seedling tissue, etiolated seedlings grown for 5 days at 26°C were transferred to the light [500 $\mu E \cdot m^{-2} \cdot sec^{-1}$; 1 E (einstein) = 1 mol of photons] for 6–8 hr. Shoots were excised above the coleoptilar node, the coleoptile was removed, and leaf tissue (1–2 g) was frozen in liquid N_2. Frozen tissue was homogenized in liquid N_2 in a mortar and extracted at a ratio of 4 ml of 0.1 M Tricine·KOH, pH 8.3/0.3 M glycerol/15 mM $NaHCO_3$/2 mM EDTA/5 mM DTT/0.2 mM phenylmethylsulfonyl fluoride per gram fresh weight. Seedling homogenates were desalted as described above in the same equilibration buffer except that DTT was reduced to 1 mM.

ACCase assays were conducted as described (10). Sethoxydim and haloxyfop were added 1 min prior to initiation of the ACCase reaction. A previous study confirmed that the ^{14}C-labeled product from this reaction comigrated with malonyl-CoA (9). Protein content of the extracts was determined according to Smith *et al.* (13). Relative ACCase activity was expressed as a fraction of the untreated enzyme activity from each line. The means of duplicate assays performed on the three extractions of each tissue culture were averaged. ACCase activity was determined in duplicate on extracts from seedlings of two homozygous mutant and two wild-type families identified as uniformly sethoxydim-tolerant or -susceptible after two generations of selfing two different regenerated plants.

RESULTS

Selection for Herbicide Tolerance. A maize tissue culture, S2, selected for growth in the presence of sethoxydim also was tolerant to haloxyfop (Fig. 1). Based on determinations

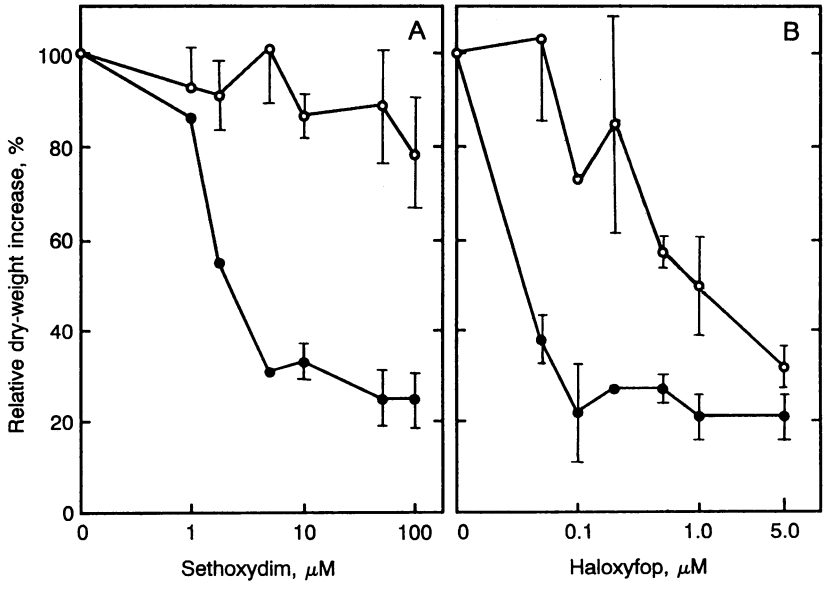

FIG. 1. Effect of sethoxydim (*A*) and haloxyfop (*B*) on growth of herbicide-tolerant S2 (○) and nonselected wild-type (●) maize tissue cultures. The herbicide concentrations inhibiting dry-weight accumulation by 50% were >100 μM sethoxydim and 0.82 μM haloxyfop for the S2 tissue culture and 2.6 μM sethoxydim and 0.04 μM haloxyfop for the wild-type tissue culture. Data are means ± SE. Where not shown, the SE is within the symbol. Dry-weight increases determined for the nonselected and S2 tissue cultures grown in the absence of herbicide were 240 ± 25.3 and 298 ± 1.2 mg for *A* and 224 ± 16.2 and 205 ± 55.2 mg for *B*, respectively.

of the herbicide concentrations required to reduce dry-weight increase of the tissue cultures by 50%, S2 exhibited a >40-fold increase in tolerance to sethoxydim and a 20-fold increase in tolerance to haloxyfop compared to the nonselected wild-type tissue culture.

Herbicide Tolerance in Regenerated Plants. Fertile plants were regenerated from S2 tissue cultures, allowing us to determine that herbicide tolerance was expressed on the whole-plant level. S2 plants survived and grew to maturity following herbicide treatments of up to 0.44 kg of sethoxydim per ha, which is twice the standard field rate for annual grass weed control (Table 1). However, the tolerance of the regenerated S2 plants was not complete, since leaves emerging from the whorl 10 days after treatment with 0.22 kg of sethoxydim per ha exhibited herbicide injury characterized by chlorotic bleaching. Wild-type plants regenerated from the nonselected callus were killed by 0.05 kg of sethoxydim per ha. This rate also was lethal to seed-grown plants of A188 and B73, the progenitor inbreds of the hybrid tissue culture, and A619, A632, A641, A661, A665, B37, R806, and W153R (data not shown). Similar to the tissue culture results, the greater lethality of haloxyfop also was observed in plants treated with haloxyfop. Wild-type plants were injured and killed by application of 0.005 and 0.01 kg of haloxyfop per ha, respectively (data not shown). Regenerated S2 plants survived but were injured by treatment with 0.01 kg of haloxyfop per ha and were killed by treatment with 0.05 kg of herbicide per ha.

Inheritance of Herbicide Tolerance. Regenerated S2 plants (R_0) were self-pollinated and were test- and backcrossed to susceptible wild-type inbreds. Progeny were treated with 0.44 kg of sethoxydim per ha in the greenhouse and 0.22 kg per ha in the field to determine the inheritance of the tolerance trait. These rates killed all progeny of selfed plants regenerated from nonselected tissue cultures (Fig. 2*A*). Selfed progeny of S2 R_0 plants treated with sethoxydim in the greenhouse segregated into three phenotypic classes of herbicide tolerance (Fig. 2*B*). One class was as susceptible as wild-type plants and was killed; another class exhibited herbicide injury similar to that of the R_0 plants but was not killed; and another class of tolerant plants was uninjured. The segregation ratio of these three classes among selfed progeny of 13 R_0 plants approximated a 1:2:1 ratio for susceptible/injured/tolerant progeny, indicating segregation of a single partially dominant mutation and that the S2 tissue culture and R_0 plants were heterozygous for the mutation conferring herbicide tolerance (Table 1). Injured plants that were subsequently selfed gave rise to progeny that segregated for tolerance, confirming the association between nonlethal herbicide injury and heterozygosity for tolerance. All selfed progeny of noninjured (tolerant) plants expressed the tolerance trait, indicating that these plants were homozygous for the trait. Homozygous tolerant R_2 plants were not injured by treatment with 0.88 kg of sethoxydim per ha in the field (4 times the field rate used to control annual grass weeds),

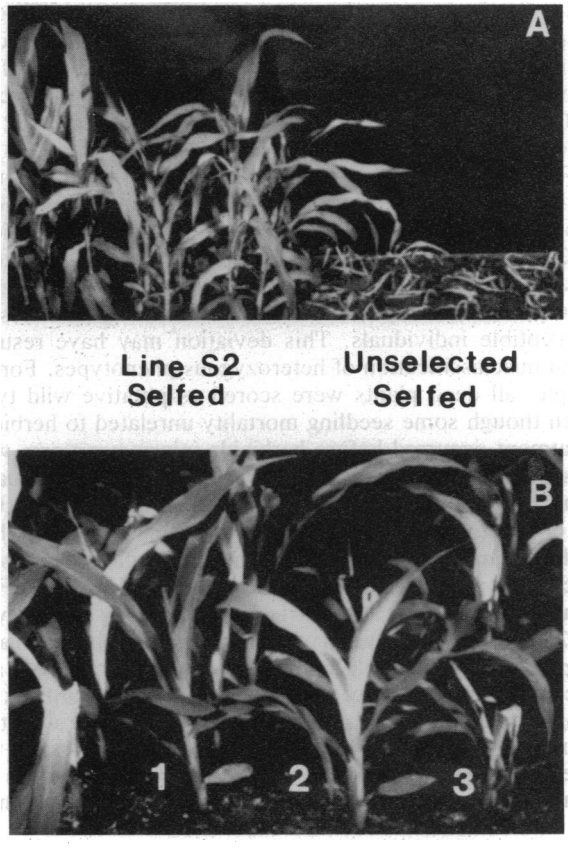

FIG. 2. (*A*) Effect of sethoxydim on progeny of selfed plants regenerated from nonselected and selected tissue cultures shown 14 days after herbicide treatment in greenhouse. (*B*) Segregation of sethoxydim injury classes observed in progeny of selfed herbicide-tolerant plants 7 days after herbicide treatment. Tolerant plants (class 1) exhibited no injury, chlorotic plants (class 2) survived herbicide treatment, and susceptible plants (class 3) were killed.

Table 1. Expression and inheritance of sethoxydim tolerance in regenerated maize plants and their progeny with or without selection for sethoxydim tolerance in tissue culture

Generation and cross	No. of crosses	No. of plants			Expected segregation ratio	*P*
		Killed	Injured	Uninjured		
Regenerated (R_0) plants						
Nonselected	—	16	0	0		—
Selected (S2)	—	0	16	0		—
Selfed (R_1) progeny of R_0						
Nonselected	13	279	0	0		—
S2	13	179	296	171	1:2:1	>0.05
Testcross progeny of R_0						
Nonselected × WT*	2	55	0	0		—
WT × nonselected	5	126	0	0		—
S2 × WT	3	71	40	0	1:1	<0.05
WT × S2	4	186	131	0	1:1	<0.05
WT × S2†	3	58	52	0	1:1	>0.50
Backcross progeny						
WT × (WT × S2)†	4	65	72	0	1:1	>0.50

*Wild-type (WT) inbreds included A619, A641, A661, A665, A188, R806, and W153R.
†Tests conducted in field with 0.22 kg of sethoxydim per ha instead of greenhouse with 0.44 kg of sethoxydim per ha.

indicating increased tolerance compared with heterozygous R_0 plants. The homozygous plants also exhibited tolerance to haloxyfop. Furthermore, the homozygous mutant plants showed no obvious alterations in stature or seed production following herbicide treatment compared to untreated S2 and wild-type maize plants.

Progeny of reciprocal testcrosses of R_0 plants to wild-type maize inbreds segregated for susceptible and injured phenotypes (Table 1). These data also indicated that herbicide tolerance was controlled by a single dominant nuclear gene; however, segregation in the greenhouse deviated significantly from the expected 1:1 ratio because of an excess of susceptible individuals. This deviation may have resulted from misclassification of heterozygous phenotypes. For example, all dead plants were scored as putative wild types even though some seedling mortality unrelated to herbicide treatment occurred before herbicide injury symptoms were observed in the majority of susceptible plants. Additionally, some heterozygous plants may have been susceptible to 0.44 kg of sethoxydim per ha applied in the greenhouse, where sethoxydim was expected to be most efficacious because of decreased degradation of the herbicide by UV light. Field evaluation of segregation in testcross progeny treated with 0.22 kg per ha sethoxydim resulted in an improved fit to a 1:1 segregation ratio (Table 1). A 1:1 segregation ratio was also accepted for progeny of the first backcross of plants exhibiting herbicide injury to four different susceptible wild-type inbreds. These data further support our conclusion that tolerance was conferred by a partially dominant mutation and demonstrate that the trait can be incorporated into commercially important inbreds for the development of herbicide-tolerant hybrids.

Altered ACCase in Tolerant Tissue Cultures and Plants. Specific activities of ACCase of the wild-type and tolerant S2 tissue cultures were similar in the absence of herbicide. ACCase activity of S2 tissue cultures required 11-fold more sethoxydim and 6-fold more haloxyfop for 50% inhibition than ACCase from the wild-type tissue cultures (Fig. 3 *A* and *B*). These results closely paralleled the tolerance of the S2 tissue cultures to the two herbicides. ACCase activities of wild-type and homozygous S2 seedlings also were similar in the absence of the herbicides. However, >100-fold more sethoxydim and 16-fold more haloxyfop were needed for 50% inhibition of ACCase activity of S2 seedlings as compared to wild-type ACCase (Fig. 3 *C* and *D*).

DISCUSSION

A partially dominant mutation conferring tolerance to sethoxydim and haloxyfop in maize, normally a susceptible species, was selected from tissue culture. The mutation presumably arose from tissue culture-induced genetic variation, since the callus was not mutagenized prior to selection. Maize plants regenerated from tissue culture often exhibit high frequencies of genetic and chromosomal changes (14). Selection of tissue cultures for growth in the presence of herbicide appeared to be necessary for isolation of the herbicide-tolerance mutation because plants regenerated from nonselected tissue cultures were not herbicide-tolerant. Furthermore, the parental genotypes of the tissue culture and several other maize inbreds

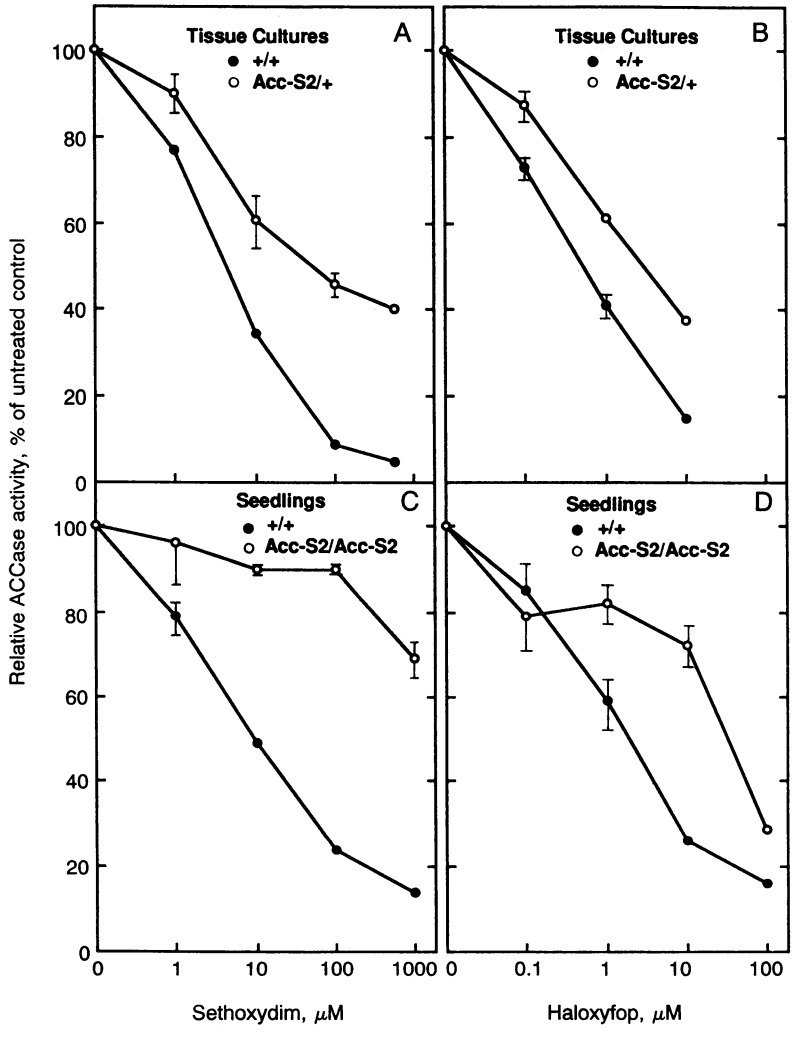

FIG. 3. Effect of sethoxydim and haloxyfop on ACCase activity from nonselected wild-type (●) and heterozygous herbicide-tolerant (○) tissue cultures (*A* and *B*) and wild-type (●) and homozygous mutant (○) seedlings (*C* and *D*). Data are means ± SE. Where not shown, the SE is within the symbol. Mean ACCase activities determined from the nonselected and selected tissue cultures in *A* and *B* were 4.2 ± 0.8 and 4.5 ± 1.0 nmol of HCO_3^- incorporated per mg of protein per min, respectively. Herbicide concentrations inhibiting 50% of the ACCase activity determined from *A* and *B* were 7 and 77 μM sethoxydim and 0.7 and 4.1 μM haloxyfop for wild-type and S2 tissue cultures, respectively. Enzyme activities for wild-type and homozygous mutant seedlings in *C* and *D* were 52 ± 2 and 58 ± 4 nmol per mg of protein per min, respectively. Herbicide concentrations inhibiting 50% of the ACCase activity determined from *C* and *D* were 10 and >1000 μM sethoxydim and 3.5 and 56 μM haloxyfop for wild-type and S2 seedlings, respectively.

tested were susceptible to sethoxydim and haloxyfop. In addition to S2, we have isolated and characterized four other partially dominant herbicide-tolerance mutations by using tissue culture selection.

Tolerance to cyclohexanedione and aryloxyphenoxypropionate herbicides has been shown to be conferred either by herbicide-insensitive forms of ACCase as in dicots (1–6) and red fescue (9) or by increases in herbicide-sensitive ACCase activity in herbicide-tolerant maize cell cultures (10). It has been proposed that detoxification of cyclohexanedione and aryloxyphenoxypropionate herbicides may account for herbicide tolerance detected in certain *Lolium rigidum* biotypes (15). Of these potential tolerance mechanisms, a mutation altering maize ACCase to be less sensitive to herbicide inhibition appears to confer herbicide tolerance in S2. Homozygous progeny of S2 plants that were tolerant to both sethoxydim and haloxyfop also exhibited ACCase activity that had reduced sensitivity to inhibition by both herbicides. Susceptible progeny had ACCase activity with wild-type herbicide sensitivity, and there were no differences in ACCase activity levels between susceptible and tolerant tissue cultures or seedlings. Partially purified ACCase isolated from homozygous mutant S2 seedlings also exhibited reduced sensitivity to herbicide inhibition (data not shown), providing further evidence that alterations in ACCase and not herbicide detoxification conferred tolerance in S2 plants. Cosegregation of an altered form of ACCase with the herbicide-tolerance trait indicated that reduction in herbicide inhibition of ACCase activity confers tolerance to sethoxydim and haloxyfop in maize. These results confirm other reports (1–6, 9) that ACCase is the site of action of cyclohexanedione and aryloxyphenoxypropionate herbicides in susceptible monocotyledonous species.

The mutation causing a reduction in herbicide inhibition of ACCase most likely occurred in the structural gene for the enzyme. An altered herbicide-insensitive form of ACCase would be expected to confer partially dominant herbicide tolerance. Maize ACCase is thought to be a homodimer of 220-kDa subunits (16). If this structural model is correct, the increased herbicide tolerance of homozygous mutant progeny of S2 compared to the heterozygous regenerated S2 plants and testcross progeny would be expected due to complete expression of homodimeric mutant ACCase in homozygous plants. Similar to our results, altered sulfonylurea-insensitive forms of acetolactate synthase that were selected from tobacco tissue cultures also were inherited as partially dominant or dominant mutations (17, 18).

Cyclohexanedione and aryloxyphenoxypropionate herbicides are mutually exclusive noncompetitive inhibitors of maize ACCase, suggesting that both herbicides bind the enzyme at the same site (19). S2 and three other selected herbicide-tolerant maize mutants also were tolerant to both sethoxydim and haloxyfop and exhibited an altered form of ACCase with reduced sensitivity to both herbicides (data not shown). However, a mutant selected for growth on haloxyfop had increased tolerance to haloxyfop but little tolerance to sethoxydim; it exhibited an altered ACCase that primarily had reduced sensitivity to haloxyfop (data not shown). It will be of interest to determine the genetic relationships among these different herbicide-tolerant phenotypes and to confirm that the mutation(s) occur in the structural gene of ACCase at the DNA sequence level. Based on our observations of cosegregation of herbicide tolerance and an altered form of ACCase that exhibits reduced herbicide inhibition, we have designated the S2 mutant allele as *Acc-S2*.

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