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Allelic mutations in acetyl-coenzyme A carboxylase confer herbicide tolerance in maize *

L. C. Marshall **, D. A. Somers ***, P. D. Dotray, B. G. Gengenbach, D. L. Wyse and J. W. Gronwald

Department of Agronomy and Plant Genetics, University of Minnesota and Plant Science Research Unit, U.S. Department of Agriculture, Agriculture Research Service, St. Paul, MN 55108 USA

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Summary. The genetic relationship between acetyl-coenzyme A carboxylase (ACCase; EC 6.4.1.2.) activity and herbicide tolerance was determined for five maize (*Zea mays* L.) mutants regenerated from tissue cultures selected for tolerance to the ACCase-inhibiting herbicides, sethoxydim and haloxyfop. Herbicide tolerance in each mutant was inherited as a partially dominant, nuclear mutation. Allelism tests indicated that the five mutations were allelic. Three distinguishable herbicide tolerance phenotypes were differentiated among the five mutants. Seedling tolerance to herbicide treatments cosegregated with reduced inhibition of seedling leaf ACCase activity by sethoxydim and haloxyfop demonstrating that alterations of ACCase conferred herbicide tolerance. Therefore, we propose that at least three, and possible five, new alleles of the maize ACCase structural gene (*Acc1*) were identified based on their differential response to sethoxydim and haloxyfop. The group represented by *Acc1-S1*, *Acc1-S2* and *Acc1-S3* alleles, which had similar phenotypes, exhibited tolerance to high rates of sethoxydim and haloxyfop. The *Acc1-H1* allele lacked sethoxydim tolerance but was tolerant to haloxyfop, whereas the *Acc1-H2* allele had intermediate tolerance to sethoxydim but was tolerant to haloxyfop. Differences in tolerance to the two herbicides among mutants homozygous for different *Acc1* alleles suggested that sites on ACCase that interact with the different herbicides do not completely overlap. These mutations in maize ACCase should prove useful in characterization of the regulatory role of ACCase in fatty acid biosynthesis and in development of herbicide-tolerant maize germplasm.

Key words: Tissue culture mutant selection – Herbicide tolerance – Fatty acid biosynthesis – Acetyl-CoA carboxylase – maize

Abbreviations and definitions: ACCase, Acetyl-coenzyme A carboxylase, EC 6.4.1.2.; *ALS*, acetolactate synthase; I_{50}, concentration of herbicide that inhibits ACCase activity by 50% of uninhibited value; R_0 to R_4, genetic nomenclature for tissue culture-derived plants where R_0 = regenerated plant and R_1 = first generation progeny from R_0

Introduction

Mutations affecting enzymes in essential biosynthesis pathways are useful in elucidating the genetics and biochemistry of fundamental plant processes. Some herbicides inhibit critical biosynthesis enzymes and thus can be used as positive selective agents to identify mutations in genes affecting the target site enzyme. We have used this approach to select for mutations in the structural gene(s) for the enzyme ACCase in maize (Parker et al. 1990 a, b). ACCase catalyzes the carboxylation of acetyl-CoA to produce malonyl-CoA, a precursor of fatty acid biosynthesis (Stumpf 1980; Harwood 1988). Recent evidence suggests that ACCase may be a rate-limiting step

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** *Present address:* Holden's Foundation Seed, P.O. Box 839, Williamsburg, IA 52361
*** To whom correspondence should be addressed
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in *de novo* fatty acid synthesis (Parker et al. 1990b; Post-Beittenmiller et al. 1991). Maize genotypes currently grown in production fields are susceptible to two classes of ACCase-inhibiting herbicides: the cyclohexanediones (e.g. sethoxydim) and the aryloxyphenoxypropionates (e.g. haloxyfop). ACCase activity is inhibited by these foliarly applied herbicides in susceptible monocotyledonous plants, whereas ACCase in dicots is unaffected (Burton et al. 1987, 1989, 1991; Focke and Lichtenthaler 1987; Rendina and Felts 1988; Rendina et al. 1988; Secor and Cseke 1988; Stoltenberg et al. 1989). Cessation of fatty acid synthesis due to herbicide inhibition of malonyl-CoA production is presumed to be the cause of the lethality of the ACCase-inhibiting herbicides (Rendina et al. 1988; Burton et al. 1989).

In our previous studies, tissue culture selection was used to isolate sethoxydim- and haloxyfop-tolerant maize tissue cultures (Parker et al. 1990a, b). Herbicide-tolerant maize plants were regenerated from three tissue cultures surviving sethoxydim selection (S1, S2 and S3) and two tissue cultures surviving haloxyfop selection (H1 and H2) (Parker 1990). Segregation for tolerance in the progeny of each mutant indicated that the tissue cultures were heterozygous for partially dominant, nuclear mutations conferring herbicide tolerance (Parker 1989; Parker et al. 1990a; Marshall 1990). ACCase activity in extracts from one of the sethoxydim-tolerant tissue cultures (S2) and its derivative homozygous tolerant seedlings exhibited decreased herbicide inhibition by both sethoxydim and haloxyfop when compared to wildtype ACCase. This association between herbicide tolerance and altered ACCase in the S2 mutant suggested that an alteration in ACCase conferred herbicide tolerance (Parker et al. 1990a).

The objective of this study was to determine the gene-enzyme relationship(s) conferring herbicide tolerance in the five maize mutants. Whole plant herbicide tolerance cosegregated with a comparable reduction in sethoxydim and haloxyfop inhibition of ACCase extracted from homozygous tolerant progeny of each mutant. Three different herbicide-tolerant phenotypes were distinguished among the mutants, and all appeared to be allelic indicating that at least three new alleles of maize ACCase were identified.

Materials and methods

Plant materials

Tissue culture lines from F_1 or F_2 maize (*Zea mays* L.) embryos derived from the cross A188 × B73 and selected for tolerance to sethoxydim (S1, S2, S3) or haloxyfop (H1, H2) were used to regenerate plants that provided seed of the mutant lines (Parker 1990; Parker et al. 1990b). S1, S2 and H1 were independently isolated from the same F_1 tissue culture line, whereas S3 and H2 were isolated from a F_2 tissue culture.

Herbicide applications

Foliar applications of the herbicides were made to greenhouse- or field-grown seedlings at the three- to five-leaf stage. Herbicides were applied in a spray volume of 187 l/ha that included 2.3 l/ha crop oil concentrate to enhance uptake. Sethoxydim and haloxyfop were applied using commercial formulations (Poast from BASF Corp and Verdict from Dow Elanco, respectively). Visual observations were recorded 14–21 days after herbicide treatments.

Herbicide tolerance

For each mutant, two homozygous tolerant R_4 families were derived from two different regenerated plants and two heterozygous F_1 families were obtained from crosses of R_3 homozygous tolerant lines with the susceptible inbreds A188 and A619. Sethoxydim and haloxyfop each were applied at rates ranging from 0.0034 to 7.0 kg/ha. For each genotype a five-rate doubling series was used to determine herbicide lethality. Because of the large differences between wildtype and mutants in herbicide susceptibility, the ranges of herbicide application rates were varied. Two five plant/pot replicates of each family were evaluated for each rate. Herbicide injury symptoms were scored visually 21 days after herbicide treatment. The lethal rate was considered to be the lowest application rate resulting in death of all seedlings in the four replications of each homozygous tolerant genotype or in the eight replications of the heterozygous combinations.

Allelic relationships among mutants

All F_1 combinations (no reciprocals) between S1, S2, S3, H1 and H2 homozygotes were made, and these F_1s were test crossed to susceptible inbred lines. The testcross progeny were grown in the greenhouse and treated with the herbicides. In Experiment 1, progeny from testcrosses with each of the ten F_1 combinations were treated with 0.05 kg/ha sethoxydim for combinations involving only S lines or with 0.009 kg/ha haloxyfop for combinations involving either of the H lines. In Experiment 2, a selected subset of the F_1 combinations was tested that used testcross families that were separately derived from those used in Experiment 1. Testcross progeny of S lines were treated with 0.03 kg/ha sethoxydim, and H1 and H2 progeny were treated with 0.009 kg/ha haloxyfop. In each experiment, known susceptible and heterozygous plants were included as controls.

ACCase activity

Seedlings from the same (or sister) families used in the evaluation of seedling tolerance were grown in a growth chamber at 26 °C under a 13-h daylength. Tissue from about 24 plants was bulked for each homozygous tolerant and heterozygous family, and for the susceptible inbreds A188, A619 and B73. Shoots were harvested 6–9 days after planting, the coleoptile and first leaf were discarded and a 5-cm segment from the base of the shoot to just above the whorl was collected. The collected leaf tissue was flash frozen in liquid nitrogen and stored at −70 C for up to 3 weeks before extraction.

Extracts were prepared by pulverizing the frozen leaf tissue in liquid nitrogen, followed by addition of 5 ml cold extraction buffer (0.1 *M* tricine-KOH pH 8.3, 0.3 *M* glycerol, 15 m*M* $NaHCO_3$, 2 m*M* EDTA, 5 m*M* DTT and 0.2 m*M* phenylmethylsulfonyl fluoride) per gram fresh weight tissue. The mixture was held on ice for about 10 min with occasional grinding, followed by 2 min of continuous grinding with a pinch of washed sand. The homogenate was filtered through Miracloth and centrifuged at 24,000 *g* for 30 min. The supernatant was desalted on a Sephadex G-25 column (1.5 × 5 cm) equilibrated

with elution buffer (0.1 M tricine-KOH pH 8.3, 0.1 M glycerol, 1 mM DTT) and stored in liquid nitrogen. Aliquots of these desalted extracts were used in enzyme assays.

Acetyl-CoA carboxylase activity was assayed by measuring the incorporation of $H[^{14}C]O_3^-$ into an acid-stable fraction (Parker et al. 1990). Assays were conducted at saturating substrate concentrations at 30 °C in a 200-µl final volume containing 50 µl extract (30–70 µg protein) in 100 mM tricine-KOH pH 8.3, 300 mM glycerol, 0.25 mM DTT, 5 mM KCL, 1 mM ATP, 2.5 mM $MgCl_2$ and 15 mM $NaHCO_3$ (0.6 mCi/mmole). The reaction was initiated with the addition of 0.5 mM acetyl-CoA and stopped after 5 min by the addition of 30 µl 4 N HCl. The mean DPM for three replicates (representing subsamples of the same extract) were determined for background, control (no herbicide) and herbicide-containing reaction mixtures for each extract. Protein concentrations in the extracts were determined (Bradford 1976) and ACCase specific activities were calculated.

Inhibition of the ACCase activity in each extract was determined for sethoxydim concentrations ranging from 1 to 4,000 µM and haloxyfop concentrations ranging from 0.1 to 500 µM. At each concentration, data for the two homozygous tolerant families, the two heterozygous families and the three susceptible inbreds were individually averaged to represent the responses of each tolerance genotype.

Plots of the relative ACCase activity versus the log of herbicide concentration were used to estimate I_{50} values. Pooled error estimates were obtained separately for sethoxydim and haloxyfop inhibition data using corresponding families of the same tolerance genotype (within each mutant) as replicates for variance estimates.

Cosegregation of plant and ACCase tolerance

For each mutant, homozygous tolerant and homozygous susceptible families derived from R_0 (heterozygous) plants were classified by evaluating plant responses to the herbicides. Enzyme extracts from these families were assayed for ACCase activity (% of no herbicide control) at herbicide concentrations near or greater than the susceptible wildtype I_{50} value. Activities in tolerant and susceptible extracts included in the same experiments were compared. ACCase activity was considered tolerant if when assayed in the presence of herbicide, it was at least 20% greater than the inhibited wildtype ACCase activity. For each of the mutant lines, tolerant lines tracing back to at least two different R_0 plants were evaluated.

Results

Herbicide tolerance

Previous characterization of the five mutants indicated that herbicide tolerance was controlled by partially dominant mutations in single nuclear gene(s) (Parker 1989; Parker et al. 1990a; Marshall 1990). To further characterize the mutant phenotypes, we determined the herbicide application rates that were lethal to wildtype, homozygous tolerant and heterozygous tolerant seedlings (Table 1). The five mutants could be differentiated into three phenotypes based on differences in tolerance to sethoxydim and haloxyfop. Homozygous seedlings of S2 and S3 exhibited only slightly bleached leaves and a slight reduction in plant height, but were not killed at 7 kg/ha sethoxydim, which is at least a 127-fold increase over the lethal rate for wildtype plants. S2 and S3 lines also exhibited cross tolerance to haloxyfop. Homozygous S1 seedlings exhibited herbicide tolerance similar to that of S2 and S3 (data not shown). Heterozygotes derived from crosses of the S lines with the wildtype exhibited a tolerance to both herbicides that was intermediate between wildtype and homozygous tolerant seedlings. In contrast to the S lines, a second distinctive phenotype was represented by H1 homozygous seedlings that exhibited little or no sethoxydim tolerance but significant haloxyfop tolerance. Heterozygotes with the H1 lines exhibited haloxyfop tolerance that was similar to that of the homozygous H1 seedlings. H2 seedlings represented the third phenotype, which exhibited about 16-fold increases in tolerance to sethoxydim and haloxyfop compared to the wildtype. The H2 heterozygotes exhibited a tolerance to both herbicides that was intermediate between wildtype and homozygous tolerant seedlings.

Table 1. Herbicide application rates lethal to greenhouse-grown seedlings of susceptible wildtype, and homozygous and heterozygous tolerant maize lines

Genotype	Lethal rates (kg/ha)	
	Sethoxydim	Haloxyfop
Wildtype[a]	0.055	0.014
Homozygous tolerant[b]		
S2	$>$7.0	0.22
S3	$>$7.0	0.22
H1	0.055	0.11
H2	0.88	0.22
Heterozygous tolerant[c]		
Inbred × S2	$\geq$1.8	0.11
Inbred × S3	$\geq$1.8	0.11
Inbred × H1	0.055	0.11
Inbred × H2	0.22	0.11

[a] Average response of A188 and A619
[b] Average response of two families per mutant line
[c] Average response of A188- and A619-derived F_1 families

The two families of each mutant, derived from different R_0 plants and representing independent derivations from the parental A188 × B73 cross, had similar responses to the herbicides (data not shown). Herbicide lethality of heterozygotes of each mutant from the A188 cross was similar to that of the A619 cross. This suggested that background effects did not play a large role in modifying herbicide tolerance. However, injury symptoms at sublethal rates (data not shown) indicated there was some tendency for the A188 inbred plants to be slightly more susceptible to both of the herbicides than A619 inbred plants. This tendency was also suggested by a few comparisons between A188- and A619-derived heterozygotes. Perhaps modification of plant-level tolerance

by genetic background was more prevalent for wildtype or heterozygous plants than for homozygous tolerant plants.

Allelic relationships among mutants

To determine allelic relationships among the mutations, F_1s made between the different homozygous mutants were crossed to herbicide-susceptible inbreds. The testcross progeny were treated with rates of herbicides intended to kill wildtype plants but allow heterozygous plants to survive. If the mutant alleles from the different mutant lines were at the same locus, then all testcross progeny would have had one or the other mutant allele contributed by the F_1 parent and would have been tolerant to the herbicide rates used. No susceptible plants were found in a total of 260 testcross progeny from crosses among S1, S2 and S3 (Table 2). Interpretation of these results was straightforward because all S line heterozygous plants included as controls in these tests survived the herbicide treatments as expected, and all inbred wildtype plants died when treated with sethoxydim (data not shown). Additionally, no haloxyfop-susceptible plants were found in a total of 190 testcross progeny from H1 combinations with S1, S2 or S3, indicating that the H1 mutation is allelic to S1, S2 and S3 mutations (Table 2).

Testcrosses involving H1 and H2 alleles were more difficult to interpret. The heterozygous controls for H1 and H2 were injured, and some were killed (data not shown) with the haloxyfop rate (0.009 kg/ha) that killed most, but not all, wildtype control plants. Four susceptible plants were found in a total of 310 testcross progeny from combinations of H2 and the S lines. This frequency was clearly different from the 77 susceptible progeny expected if the H2 allele was at an independent locus. It seems most likely that these deaths occurred due to variability in response of the H2 heterozygous plants, but the possibility that the haloxyfop-susceptible plants represented recombination between two closely linked loci cannot be ruled out. The 5 haloxyfop-susceptible plants among 102 testcross progeny from the H1 and H2 combination also represented a clear difference from the 25 susceptible expected for independent loci, and again may be explained by a variable response of H2 or H1 heterozygous plants. In the testcrosses involving H2, all of the plants that died were from crosses to the inbred A188 (Table 2). A188 was slightly more susceptible than other

Table 2. Allelism tests to detect seedling lethality from herbicide treatment in progeny from testcrosses of various mutant F_1 combinations to several inbred backgrounds

F_1 mutant genotype	Experiment 1			Experiment 2			Summary	
	Inbred	Dead	Total tested	Inbred	Dead	Total tested	Dead	Total tested
S1, S2	A619	0	50[b]	A619	0	55[e]		
S1, S3	A619	0	50[b]	–	–	–		
S2, S3	W153R	0	50[b]	A641	0	55[e]		
All S lines							0	260
H1, S1	A619	0	50[c]	–	–	–		
H1, S2	A619	0	50[c]	A619	0	40[c]		
H1, S3	A619	0	50[c]	–	–	–		
H1 and all S lines							0	190
H2, S1	A188	0	50[c]	–	–	–		
H2, S2	A619	0	50[c]	A188	2[d]	84[f]		
H2, S3	A188	2[a]	50[c]	A641	0	76[f]		
H2 and all S lines							4	310
H1, H2	A188	0	49[c]	A188	5[a]	53[c]	5	102

[a] Dead plants after haloxyfop treatment
[b] Treated with 0.05 kg/ha sethoxydim
[c] Treated with 0.009 kg/ha haloxyfop
[d] One dead plant after sethoxydim treatment, 1 dead plant after haloxyfop treatment
[e] Treated with 0.03 kg/ha sethoxydim
[f] Subsamples of each progeny treated with 0.03 kg/ha sethoxydim or 0.009 kg/ha haloxyfop

inbreds, which may have resulted in a slightly lower tolerance in heterozygotes derived from A188. The cause of rare deaths cannot be conclusively interpreted. However, the most likely interpretation is that the S1, S2, S3 and H1 mutations are allelic and that H2 either is allelic or linked (≤4.4 map units, if all 9 plants that died were due to recombination).

ACCase activity

ACCase activity was determined from wildtype and mutant seedling extracts for further comparison of the mutants at the site of herbicide inhibition. In the absence of herbicide, ACCase activity levels for wildtype, S2, S3, H1 and H2 seedling extracts were 82, 81, 80, 61 and 57 ($LSD_{05}=11.9$) nmoles/mg protein per minute, respectively. The lower ACCase activities of H_1 and H_2 may reflect differences caused by the mutations, or may be a reflection of limited sampling. ACCase activity in extracts from the wildtype inbreds A188, B73 and A619 showed nearly identical inhibition patterns for various concentrations of both herbicides. Data were averaged across inbreds at each herbicide concentration to obtain the wildtype response curves presented in Figs. 1 and 2. From these curves, I_{50} values of 7.2 μM sethoxydim and 1.2 μM haloxyfop were calculated for ACCase extracted from wildtype maize. The ACCase activity in extracts of S2 and S3 homozygous tolerant seedlings was only slightly inhibited by sethoxydim concentrations of up to 1 mM (Fig. 1). The sethoxydim I_{50} values of 2,400 μM for S2 and 2,740 μM for S3 homozygous tolerant seedling extracts represented about 350-fold increases over the wildtype I_{50} and indicated ACCase activities that were highly tolerant to sethoxydim (Table 3). Haloxyfop I_{50} values of 24 μM for S2 and 31 μM for S3 homozygous tolerant extracts represented about 25-fold increases over the wildtype I_{50}, and indicated moderate tolerance to haloxyfop at the enzyme level (Fig. 2, Table 3). Herbicide inhibition of ACCase in extracts of homozygous tolerant S1 seedlings (data not shown) was similar to S2 and S3.

Sethoxydim inhibition of ACCase activity in the H1 homozygous tolerant extracts was similar to wildtype (Fig. 1, Table 3). However, H1 ACCase activity was clearly tolerant to haloxyfop, with an I_{50} value of 19 μM representing a 16-fold increase over the wildtype. Sethoxydim inhibition of ACCase activity in the H2 homozygous tolerant extract was less than for wildtype and H1 but more than for the S lines (Fig. 1). The sethoxydim I_{50} value of 260 μM for H2 represents a 36-fold increase over wildtype (Table 3). Haloxyfop inhibition for H2 ACCase activity was similar to that for S2 and S3 extracts (Fig. 2).

Extracts from heterozygotes from each of the mutant lines displayed an intermediate level of ACCase tolerance in all cases where the ACCase activity from homozygous

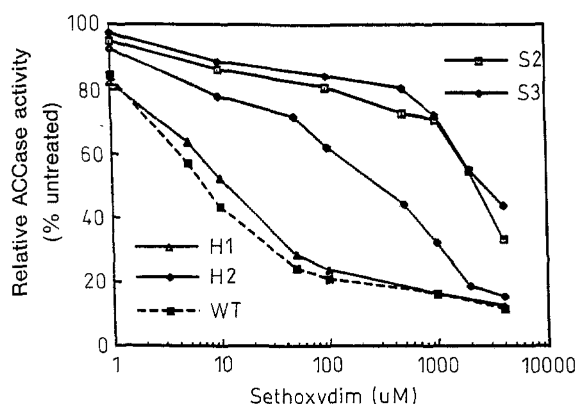

Fig. 1. Sethoxydim inhibition of acetyl-coenzyme A carboxylase extracted from homozygous tolerant mutant and susceptible wildtype seedlings. Pooled LSD 0.05 = 6.4 percentage points

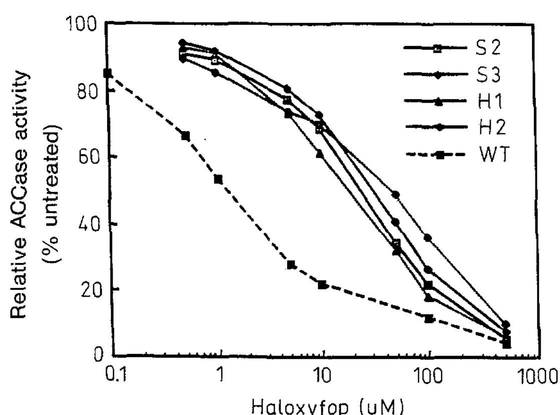

Fig. 2. Haloxyfop inhibition of acetyl-coenzyme A carboxylase extracted from homozygous tolerant mutant and susceptible wildtype seedlings. Pooled LSD 0.05 = 3.3 percentage points

Table 3. Herbicide seedling lethality and herbicide inhibition of ACCase activity extracted from homozygous and heterozygous tolerant lines expressed as fold increases over values determined for wildtype seedlings

Line	Herbicide lethality		ACCase I_{50}	
	Sethoxydim	Haloxyfop	Sethoxydim	Haloxyfop
	Fold increase relative to wildtype			
Homozygous tolerant				
S2	>127	16	350	23
S3	>127	16	350	23
H1	1	8	1.6	16
H2	16	16	36	40
Heterozygous tolerant				
Inbred × S2	>33	8	36	6.6
Inbred × S3	>33	8	36	6.6
Inbred × H1	1	8	1.2	4.5
Inbred × H2	16	8	3.3	4.0

tolerant lines was clearly tolerant (Table 3). The ACCase inhibition patterns were nearly identical for different families tested within each mutant genotype. This suggests that genetic background does not play a large role in determining the ACCase inhibition pattern.

Relationship between plant and ACCase tolerance

For all of the mutants an increase in the concentration of herbicide that inhibited ACCase activity by 50% was associated with an increase in the herbicide application rate needed to kill all seedlings of that genotype (Table 3). The magnitudes of the changes in ACCase tolerance and plant tolerance were generally comparable. However, homozygous H2 ACCase tolerance to sethoxydim was nearly identical to that of heterozygous S2 and S3, while the plant sethoxydim tolerance was greater for S2 and S3 heterozygous plants compared to homozygous H2 seedlings.

Cosegregation of plant and ACCase tolerance to sethoxydim and haloxyfop

Homozygous mutant and wildtype families were derived from self-pollinations of original regenerated plants for each mutant line. Herbicide tolerance at the plant level always cosegregated with reduced inhibition of ACCase activity. Among 41 families from all five mutants, ACCase activity of each of 24 herbicide-tolerant families was at least 20 percentage points higher than ACCase activity from the 17 susceptible families at herbicide concentrations near the wildtype I_{50}. Cosegregation over several generations provides evidence that the plant and enzyme phenotypes are due to the same mutation(s) and that the mechanism for tolerance in the mutant plants is an altered form of ACCase that exhibits reduced herbicide inhibition.

Discussion

Five single gene, herbicide-tolerant mutants exhibited ACCase activity that was less inhibited by one or more of the two classes of ACCase-inhibiting herbicides than ACCase activity in wildtype (susceptible) maize. The five mutations appeared to be allelic although it is possible that susceptible progeny of crosses with H2 were recombinants between linked loci encoding ACCase. This will be difficult to verify without flanking molecular markers. Whole plant herbicide tolerance and reduced herbicide inhibition of ACCase cosegregated, and both measures of tolerance responded in parallel in all five mutants, indicating that each mutation resulted in an alteration of the herbicide-binding site(s) on the ACCase molecule.

We have obtained at least three distinct herbicide tolerance phenotypes encoded by alleles at a locus for an ACCase structural gene. As suggested by Parker et al. (1990a), we have designated the ACCase structural gene *Acc1* and the corresponding herbicide tolerant alleles *Acc1-S1, Acc1-S2, Acc1-S3, Acc1-H1* and *Acc1-H2* consistent with maize genetics nomenclature (Coe et al. 1988). All mutant phenotypes exhibited tolerance to haloxyfop, an aryloxyphenoxypropionate, but the phenotypes were distinguished by three different levels of tolerance to sethoxydim, a cyclohexanedione. *Acc1-S1, Acc1-S2* and *Acc1-S3* all exhibited high levels of tolerance to sethoxydim. *Acc1-H1* was not tolerant to sethoxydim. *Acc1-H2* exhibited intermediate sethoxydim tolerance compared to the wildtype and the S alleles and was tolerant to haloxyfop. The *Acc1-S1* and *Acc1-S2* alleles were selected from the same tissue culture and could represent reselection of the same mutational event. However, *Acc1-S3* was isolated from a different tissue culture indicating independent selection of the same phenotype. It is possible that the three *Acc1-S* alleles may differ, but differences in phenotype were not evident in the tests used. Further characterization of the kinetic properties of the ACCases from the *Acc1-S* lines, or of cross-tolerance of the plants or enzymes to different cyclohexanedione or aryloxyphenoxypropionate herbicides, may indicate subtle differences between the *Acc1-S* alleles. However, understanding the relationship between the *Acc1-S* alleles may require sequence data for the ACCase protein or gene.

Our conclusion that alterations in ACCase conferred herbicide tolerance was further supported by a number of observations. Herbicide tolerance of the maize mutants was not explained by overexpression of ACCase activity. ACCase specific activities in extracts of S2 and S3 homozygous seedlings were the same as in the wildtype, indicating that the ACCase encoded by these alleles functioned normally in our assay conditions. H1 and H2 homozygotes had ACCase specific activities that were slightly less than that of the wildtype, which may indicate that the mutations affected catalytic functions. It will be important to carefully analyze the kinetic properties of the ACCase activity in the mutants over a range of conditions, including those likely to be found in vivo, to detect any changes in catalytic efficiency associated with the mutations. The presence of some uncharacterized enzyme activity capable of metabolizing the herbicide in crude extracts of the tolerant plants and thus allowing the ACCase to avoid inhibition by herbicide is possible. Such a tolerance mechanism would also be expected to be inherited as a dominant or partially dominant trait. However, highly purified ACCase prepared from S2 homozygous tolerant seedling leaves exhibited a 175-fold increase in the I_{50} for sethoxydim compared to ACCase purified from B73 seedlings (J. Burton and J. Gronwald, unpublished data). Which was similar to our comparisons of ACCase activity from crude extracts of *Acc1-S2* and wildtype seedlings (Table 3). The purification proce-

dure would likely have removed or at least diluted a herbicide-metabolizing activity. Thus, we conclude that the mutations confer alterations in the ACCase protein structure that reduce herbicide inhibition of ACCase activity.

The mutations may occur in herbicide-binding site(s) on the ACCase molecule that can be altered without affecting the catalytic properties of the enzyme (perhaps represented by *Acc1-S2* and *Acc1-S3*) or in sites which may affect enzyme activity when altered (perhaps represented by the *Acc1-H1* and *Acc1-H2* alleles). Altered ACCase activity might have some effect on fatty acid biosynthesis or perhaps on overall plant fitness. Herbicide inhibition of susceptible ACCase has been linked to effects on the transcarboxylation function of the enzyme (Rendina et al. 1988; Burton 1991). The transcarboxylation site might be a likely site for the malonyl-CoA inhibition observed by Rendina et al. (1988). Perhaps a malonyl-CoA feed-back inhibition site and the herbicide-binding site are related, and alterations in the herbicide-binding site may concomitantly affect feedback inhibition by malonyl-CoA and ultimately affect fatty acid biosynthesis.

Different alleles for acetolactate synthase (ALS) are associated with different responses to herbicides that act by inhibiting ALS in susceptible plants (Mazur and Falco 1989). Both the sulfonylurea and imidazolinone classes of herbicides are ALS-inhibitors, and some of the ALS alleles confer cross-tolerance to both classes while others give tolerance to only one class. The alleles for ACCase also showed differences in cross-tolerance, and it is possible that these differences, as for the ALS alleles, trace to substitutions of different amino acids at critical locations in the herbicide-binding sites (Mazur and Falco 1989). Kinetic studies using ACCase that was partially purified from Black Mexican Sweet maize cell cultures suggest that herbicides from the cyclohexanedione and aryloxyphenoxypropionate classes are mutually exclusive inhibitors (Burton et al. 1991). Although the *Acc1-S1*, *Acc1-S2* and *Acc1-S3* alleles conferred cross-tolerance to sethoxydim and haloxyfop, the differences in cross-tolerance to members of the different ACCase-inhibitor chemistries observed in homozygous *Acc1-H1* and *Acc1-H2* suggested that the sites on the ACCase molecule which interact with the different herbicide chemistries do not completely overlap. Further study of the cross-tolerance of the mutant ACCases as well as DNA or protein sequence comparisons among mutant and wildtype *Acc1* alleles may shed light on the structure of the herbicide-binding sites.

The simple genetic changes conferring high levels of tolerance and the apparent retention of ACCase catalytic activity in the mutants indicate that some mutant alleles may be suitable for incorporation into germplasm used for commercial maize production. Furthermore, these mutants should prove valuable for investigations of the role of ACCase in regulating fatty acid biosynthesis. From this study and our previous ones, we have demonstrated the presence of the altered ACCase and expression of herbicide tolerance in embryogenic callus (Parker et al. 1990b) and seedling leaves and roots (Marshall 1990). These results suggest that the *Acc1* gene is expressed in vegetative tissues and is primarily involved in fatty acid biosynthesis for membrane synthesis and maintenance. We are currently using the altered forms of ACCase as polymorphic markers to determine whether the *Acc1* gene is also involved in biosynthesis of fatty acids that are incorporated into triacylglycerides during maize embryo development.

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