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Genetic and biochemical characterization of corn inbred lines tolerant to the sulfonylurea herbicide primisulfuron

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Summary. Inbred lines of corn (*Zea mays* L.) have been characterized, which exhibit differential sensitivity to the sulfonylurea herbicide primisulfuron (2-[3-(4,6-bis(difluoromethoxy)-pyrimidin-2-yl)-ureidosulfonyl]-benzoic acid methylester). When treated postemergence with 160 g a.i. per hectare, inbred 4C0 exhibited complete tolerance while inbred 4N5 was killed. The F_1 hybrid 4C0 × 4N5 was uniformly tolerant indicating dominance of the tolerance trait. The field observations correlated with laboratory tests in which seedling root growth was measured. Based on IC_{50}, inbred 4C0 was more than ten times more tolerant than inbred 4N5. In the F_2 and F_3 generations, a 3:1 segregation of tolerant and sensitive individuals was observed, consistent with tolerance being inherited as a single dominant trait. Backcrosses of heterozygous F_1 plants with the sensitive parent (4N5) yielded progeny that segreated at the expected 1:1 ratio. Backcrosses with 4C0 yielded tolerant offspring only. Inhibition characteristics of acetohydroxyacid synthase (AHAS; E.C. 4.1.3.18) were determined. The enzymes from both inbreds and their F_1 hybrid were equally sensitive and strongly inhibited by primisulfuron (IC_{50}: 7 n*M*). The fate of ^{14}C-labeled primisulfuron in seedling tissues of inbred 4C0 and the hybrid, 4C0 × 4N5, indicated rapid metabolism with a half-life ($t_{1/2}$) of approximately 3 h. On the other hand, the herbicide-sensitive inbred 4N5 was considerably slower to metabolize primisulfuron ($t_{1/2} > 24$ h). These data indicate that differential metabolism is the mechanism of tolerance to the sulfonylurea herbicide primisulfuron in tolerant corn.

Key words: Sulfonylurea – Primisulfuron – Herbicide tolerance – Acetohydroxyacid synthase – Detoxification

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Introduction

Sulfonylureas are a new class of potent low-rate herbicides (Levitt 1983). Acetohydroxyacid synthase (AHAS; E.C. 4.1.3.18), the first enzyme in the biosynthetic pathway for the branched chain amino acids valine, leucine, and isoleucine, has been identified as the target site of sulfonylurea herbicides in plants (Chaleff and Mauvais 1984). In sensitive plants, the activity of this enzyme is inhibited by nanomolar concentrations of sulfonylureas. Interestingly, this same enzyme is also the target site for two other, unrelated, classes of chemical compounds known as imidazolinone (Shaner et al. 1984) and triazolopyrimidine herbicides (Gerwick et al. 1987). The fact that animals lack the biosynthetic pathway for branched chain amino acids explains the specificity of sulfonylurea activity to plants and their low toxicity to animal and human life. Naturally tolerant plants generally possess a specific capacity to detoxify a given sulfonylurea (Sweetser et al. 1982; Hutchison et al. 1984; Neighbors and Privalle 1990). Most commonly found are hydroxylation reactions followed by glucosylation, which render the compound herbicidally inactive.

Mutants resistant to the sulfonylurea herbicides chlorsulfuron and sulfometuron methyl (DuPont Corp.) have been reported for tobacco (Chaleff and Mauvais 1984; Chaleff and Bascomb 1987), *Arabidopsis* (Haughn and Somerville 1986), *Brassica napus* (Swanson et al. 1988), and flax (Jordan and McHughen 1987). Cell cultures resistant to sulfonylurea herbicides have also been selected in vitro from *Datura innoxia* (Saxena and King 1988) and tobacco (Harms and DiMaio 1990). These mutants have shown a mode of tolerance that does not involve degradative or conjugative detoxification. Instead, the AHAS activity of the resistant mutants was found to be less sensitive to inhibition by chlorsulfuron

than that of wild-type plants (Chaleff and Mauvais 1984; Swanson et al. 1988).

Wild-type and mutant plant genes encoding AHAS have been cloned and sequenced from *Arabidopsis* and tobacco (Mazur et al. 1987) and corn (Jen et al. 1990). The specific amino acid changes resulting from three mutations in the *SuR*A and *SuR*A genes of tobacco have been determined (Lee et al. 1988; Armour et al. 1989). Transgenic plants harboring mutant AHAS genes were shown to tolerate sulfonylurea herbicides (Haughn et al. 1988; Lee et al. 1988; Wiersma et al. 1989).

Recently a new sulfonylurea herbicide, primisulfuron, has been reported which provides selective control of *Sorghum* and *Elymus* spp. in corn (Maurer et al. 1987). Tobacco cell lines resistant to primisulfuron have been obtained by in vitro selection (Harms and DiMaio 1990). Here we report studies aimed at elucidating the genetic basis and the biochemical mechanism of primisulfuron tolerance in corn.

Materials and methods

Plant material and field treatments

Preliminary research had indicated a differential response of corn inbreds 4C0 and 4N5 to primisulfuron. 4C0, 4N5, and their F_1, F_2, and backcross progenies were grown in the greenhouse (NC) and in a nursery location near Bloomington (IL). Rows of corn plants at the six-leaf stage were sprayed with a solution of primisulfuron at a rate of 160 g a.i./ha, which is about eight times higher than the recommended application rate (Maurer et al. 1987). Herbicide effects were scored in weekly intervals following application.

Chemicals

Primisulfuron (2-[3-(4,6-bis(difluoromethoxy)-pyrimidin-2-yl)-ureidosulfonyl]-benzoic acid methylester; CGA 136'872; Fig. 1) was obtained from CIBA-GEIGY Corp. (Greensboro/NC). Technical grade primisulfuron was used in a 75% water dispersable granule formulation for field application. For all other experiments, analytical grade primisulfuron was dissolved in acetone to give concentrated stock solutions, which were diluted as needed. [Phenyl-^{14}C]-labelled primisulfuron (specific activity = 60.2 μCi/mg) was obtained from CIBA-GEIGY Corp. (Greensboro/NC). Radiochemical purity, as determined by HPLC, was greater than 95%.

Seedling root growth assays

Corn seeds were surface-sterilized for 15 min using 20% commercial chlorox bleach, rinsed three times with sterile water, and placed in a row, 2.5 cm apart, on moistened autoclaved germination paper (25 × 38 cm), 2.5 cm from the top, 10 seeds per paper. A second sheet was placed on top and the papers were rolled up. Replicate rolls were placed upright in 4-l plastic beakers containing 1.5 l of sterile 1 m*M* $Ca(SO_4)_2$ solution containing 0–3000 ng/ml of primisulfuron (filter-sterilized). A transparent plastic bag was used to cover the beakers. The beakers were then incubated at 25 °C at 85% relative humidity under a 16/8 h photoperiod of 220 μE/m^2/s. Shoot and root length were measured after 7 days.

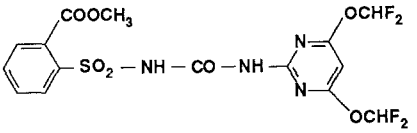

Fig. 1. Structural formula of primisulfuron

Tissue extraction and AHAS enzyme assays

Seedlings were grown hydroponically in Hoagland's nutrient solution without primisulfuron for 14 days. Shoots and roots were harvested separately, immersed in liquid nitrogen, and stored frozen at –80 °C. For extraction, 5 g of frozen tissue was ground in a Waring blender and then passed through a French pressure cell after adding 10 ml homogenization buffer containing 50 m*M* potassium phosphate buffer (pH 7.5), 1 m*M* phenylmethylsulfonylfluoride (PMSF), 20 m*M* sodium pyruvate, 5 m*M* $MgCl_2$, 1 m*M* ethylenediaminetetraacetic acid (EDTA), 5 m*M* thiamine pyrophosphate (TPP), 10 μ*M* flavin adenine dinucleotide (FAD), 10 m*M* leucine, 10 m*M* valine, 1 m*M* dithiothreitol (DTT), and 10% glycerol. The extracts were centrifuged for 10 min at 10,000 × *g*. The protein pellet from a 25%–50% ammonium sulfate cut was dissolved in column buffer containing 50 m*M* TRIS-HCl (pH 7.5), 10 μ*M* FAD, 5 m*M* $MgCl_2$, 0.1 m*M* EDTA, 1 m*M* PMSF, 1 m*M* DTT, and 10% glycerol. Root extracts were desalted on Sephadex G25 spin columns poured in 3-ml syringes. Protein concentrations were determined using the Bradford method (Bio-Rad).

The eluted extract was assayed for AHAS activity essentially as described by Muhitch et al. (1987) in a microtiter plate assay with a total reaction volume of 150 μl. The assay mixture contained 50 m*M* TRIS-HCl (pH 7.5), 10 m*M* $MgCl_2$, 10 μ*M* FAD, 70 m*M* sodium pyruvate (pH 7.0), 1 m*M* TPP, plant extract (0.1–2.0 μg protein), and 0–10,000 n*M* primisulfuron. The reaction was incubated for 1 h at 37 °C and stopped by the addition of 25 μl 2.4 *M* H_2SO_4 containing 1% creatine. Decarboxylation was carried out for 30 min at 60 °C. Color development was for 15 min at 60 °C after the addition of 10% α-naphthol dissolved in 5 *M* NaOH. Absorbance was read in a microtiter plate reader at 520 nm against no-extract and no-pyruvate blanks.

Detoxification of primisulfuron by corn seedling tissue

Seedlings were grown in sand with a 16 h light/8 h dark photoperiod at 27°/20 °C day/night temperatures. Plants were fertilized daily using a 20-10-20 general purpose fertilizer. At 3 weeks, seedlings were removed from the sand, washed, and placed in tubes containing 2,000 ng/ml primisulfuron spiked with [phenyl-^{14}C] primisulfuron in 5 m*M* potassium phosphate buffer (pH 7.0). Following incubation at 27 °C for 90 min, plants were transferred to water and incubated further. Samples consisting of five or six seedlings were taken at 0, 6, and 24 h, separated into root and shoot fractions, and frozen in liquid nitrogen. Tissue samples were stored at –80 °C until extracted. The frozen tissue was ground to a fine powder under liquid nitrogen. The powder was extracted with acetone:acetonitrile (ACN):water (70%:10%:20%) with shaking for 3 h, then centrifuged for 5 min at 3,000 × *g*. The supernatant was concentrated to 1–5 ml in vacuo at 40 °C using a rotary evaporator. The flask was rinsed with 5 ml ACN and combined with the aqueous plant extract. Following evaporation of ACN under a stream of nitrogen, the remaining aqueous extract was filtered using a 0.2-μm filter and the final volume was adjusted to 1.5 ml. The extract was subjected to reverse phase chromatography on an ODS Zorbax C18 column (4.6 × 250 mm) with a solvent system of ACN and water,

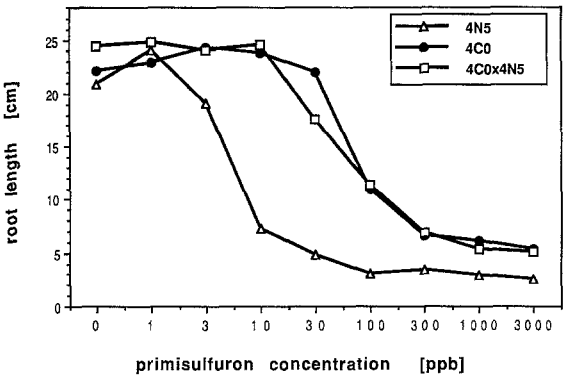

Fig. 2. Inhibition of seedling root length by primisulfuron. Each point represents the mean of 20 seedlings (ppb = ng/ml)

each acidified with 0.01% formic acid. The chromatography was carried out at a flow rate of 1.5 ml/min and a column temperature of 40 °C. The elution protocol was as follows: 5%–20% ACN (10 min), 20%–25% ACN (5 min), 25%–35% ACN (5 min), 35%–50% ACN (5 min), 50%–70% ACN (5 min), and 70%–100% ACN (5 min). Fractions were collected at 1-min intervals and radioactivity was determined by liquid scintillation counting.

Results

Field tolerance

When plants were sprayed in the field at a rate of 160 g a.i./ha of primisulfuron, inbreds 4C0 and 4N5 responded distinctly differently. Inbred 4C0 was fully tolerant, whereas 4N5 was killed within 10–14 days of the application. At the same rate, F_1 (4C0 × 4N5) plants were tolerant. Following self-pollination, F_2 progenies were grown ear-to-row in the following season. Table 1 shows a 3:1 segregation of tolerant and sensitive individuals in the F_2 generation, which indicates that primisulfuron tolerance was inherited by a single dominant gene.

Table 1. Segregation of primisulfuron-resistant and -sensitive plants in the field among individuals of F_2 progeny derived from selfed (4C0 × 4N5)

Phenotype	Genotype	No. of individuals		χ^2
		Observed	Expected	
Resistant	(R/R)	7	12.5	2.42
Resistant	(R/r)	33	24	3.37
Sensitive	(r/r)	10	12.5	0.5

Primisulfuron tolerance measured by seedling root growth assay

A dose-response curve for primisulfuron was established using seedlings of inbreds 4C0 and 4N5 and their F_1 hybrid. Root length was found to be a more suitable parameter for comparing herbicide tolerance than shoot length because of lower variability, greater absolute difference between untreated and herbicide-treated roots, and steeper dose-response interaction observed with roots. Figure 2 shows the mean root length of seedlings as a function of herbicide concentration. Inbred 4C0 and the F_1 hybrid were clearly tolerant, exhibiting IC_{50} values (herbicide concentration for half maximal root growth) of 95 ng/ml and 85 ng/ml, respectively. By contrast, inbred 4N5 was more than ten times more sensitive to primisulfuron ($IC_{50} = 7$ ng/ml).

Inheritance of primisulfuron tolerance

F_1 plants were grown to maturity and selfed to produce F_2 seed. F_1 plants were also backcrossed, reciprocally, to both inbred parents. Progeny seed was then evaluated in the seedling root growth assay for tolerance to primisulfuron. A primisulfuron concentration of 20 ng/ml was chosen for these assays because it produced the maximum difference between the tolerant and the sensitive inbred in the dose-response experiments (Fig. 2). This allowed individuals to be unambiguously categorized "tolerant" or "sensitive". Table 2 summarizes the results.

As was expected from the dose-response curves (Fig. 2) and the field observations, both inbred 4C0 and the F_1 hybrid 4C0 × 4N5 were fully tolerant of 20 ng/ml primisulfuron (Table 2), whereas the root length of inbred 4N5 was reduced by more than 80%. When the F_1 hybrid was selfed, the resulting F_2 seedlings showed a segregation of tolerant and sensitive individuals at a ratio of 3:1 (Table 2). All progeny produced from the backcross of F_1 and the tolerant inbred, 4C0, were tolerant in the root growth test (Table 2). By contrast, the progeny resulting from backcrossing the F_1 hybrid to the sensitive parental inbred 4N5 segregated as tolerant and sensitive individuals at a ratio of 1:1 (Table 2).

Random F_2 plants (from selfed F_1) were grown to maturity and selfed again. The resulting F_3 progenies were tested in the seedling root growth assay (Table 2). Of 20 progenies thus analyzed, 6 had only tolerant individuals, indicating a homozygous parent. Twelve segregated tolerant and sensitive individuals at a ratio of 3:1, indicating a heterozygous parent. Two F_3 progenies had only sensitive individuals, indicating that the parent plant was homozygous recessive for the herbicide tolerance trait.

Inhibition of acetohydroxyacid synthase by primisulfuron

In order to determine whether the primisulfuron tolerance of inbred 4C0 was based on a more tolerant AHAS as compared to the sensitive inbred 4N5, we measured AHAS activity in both inbreds and their F_1 hybrid. Fig-

Table 2. Segregation analysis of primisulfuron tolerance among corn inbreds 4C0, 4N5, and various progenies derived from them. Seedlings were scored in the seedling root growth assay (see footnote and "Materials and methods" for details)[a]

Cross	Segregation[b]				χ^2
	Observed		Expected		
	Tol.	Sens.	Tol.	Sens.	
4C0	50	0	50	0	–
4N5	0	44	0	44	–
(4C0 × 4N5)	38	0	38	0	–
(4C0 × 4N5) self	37	12	36.75	12.25	0.01
(4C0 × 4N5) × 4C0	97	2	99	0	2.04
(4C0 × 4N5) × 4N5	51	49	50	50	0.04
4N5 × (4C0 × 4N5)	22	21	21.5	21.5	0.02
F_3-1[c]	46	1	47	0	0.02
F_3-2	42	6	48	0	0.75
F_3-3	48	2	50	0	0.08
F_3-4	49	0	49	0	–
F_3-5	34	15	36.75	12.25	0.82
F_3-6	34	16	37.5	12.5	1.31
F_3-7	32	14	34.5	11.5	0.72
F_3-8	1	49	0	50	0.02
F_3-9	40	9	36.75	12.25	1.15
F_3-10	49	1	50	0	0.02
F_3-11	27	16	32.25	10.75	3.42
F_3-12	43	4	47	0	0.34
F_3-13	35	15	37.5	12.5	0.67
F_3-14	21	17	28.5	9.5	7.89
F_3-15	24	13	27.75	9.25	2.03
F_3-16	35	13	36	12	0.11
F_3-17	39	9	36	12	1.00
F_3-18	31	17	36	12	2.78
F_3-19	2	43	0	45	0.09
F_3-20	34	16	37.5	12.5	1.31

[a] Data presentation restricted by publisher; complete data available upon request from the first author

[b] The primisulfuron concentration used was 20 ng/ml; seedlings with roots >10 cm were scored "tolerant", seedlings with roots <10 cm were scored "sensitive". The root length of control seedlings (no herbicide) was 20–25 cm (Fig. 2); at 20 ng/ml the root length of seedlings scored "tolerant" was 18–25 cm, that of "sensitive" seedlings 0.5–9 cm

[c] Random seeds from selfed F_1 plants were grown to maturity and selfed to give these F_3s

Tol. – Tolerant

Sens. – Sensitive

ure 3 shows that AHAS activity of both inbreds was inhibited by primisulfuron to the same extent. The hybrid showed the same sensitivity. The enzyme exhibited half maximal activity (50% inhibition) at about 7 n*M* primisulfuron. Other herbicidal AHAS inhibitors, which indiscriminately killed all three corn genotypes in the field as well as in the seedling root growth assay, were also found to inhibit AHAS activity of 4C0, 4N5, and their F_1 at about the same level (data not shown).

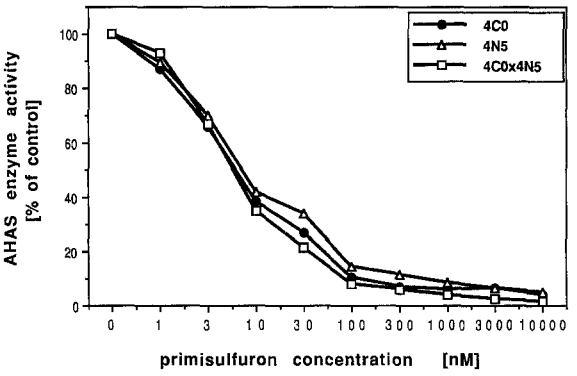

Fig. 3. Inhibition of AHAS enzyme activity by primisulfuron (mean of triplicate determinations)

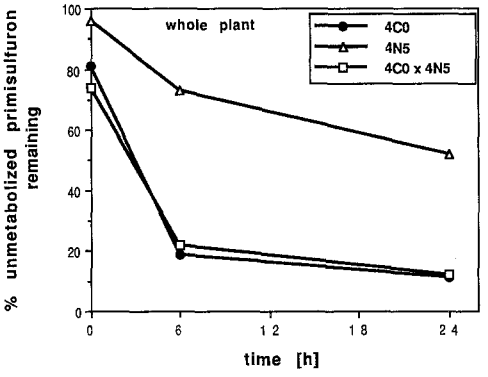

Fig. 4. Metabolism of primisulfuron in corn seedling tissues. Data represent the amount of phenyl-^{14}C-labelled primisulfuron present at various times in percent of the amount present at time $t=0$

Metabolism of phenyl-^{14}C labelled primisulfuron in seedling tissues

Young plants of inbreds 4C0 and 4N5 and their hybrid 4C0 × 4N5 were characterized for their ability to metabolize primisulfuron. Inbred 4C0, the tolerant line, as well as the F_1 hybrid showed rapid metabolism of primisulfuron (Fig. 4). Half of the parent compound was converted to a nontoxic metabolite within approximately 3 h. In contrast, at the conclusion of the experiment after 24 h, over 50% of the herbicide remained unchanged in the sensitive inbred 4N5.

Discussion

The dramatic difference observed in the field in their response to treatment with primisulfuron prompted us to perform a series of crosses between corn inbreds 4C0 and 4N5, in order to elucidate the mode of inheritance and to determine the number of genes necessary to confer primisulfuron tolerance. The results obtained in the field were

suggestive of a single dominant trait inherited by the tolerant inbred 4C0. More extensive tests using the seedling root growth assay fully confirmed the genetic model. It is worthwhile noting how extremely well the field results and the lab test correlated, although one test was scoring shoot, the other root response. The data obtained from the various crosses are consistent with the interpretation that the genetic basis of primisulfuron tolerance is a single dominant gene present in the tolerant inbred 4C0. According to this model, inbred 4N5 represents the homozygous recessive form of the gene whose phenotype is sensitivity to the herbicide. Since the direction of the crosses did not affect the herbicide tolerance of the progenies, maternal (i.e., cytoplasmic) effects can be excluded. It can be concluded, then, that primisulfuron tolerance is encoded by a nuclear gene.

Acetohydroxyacid synthase (AHAS; E.C. 4.1.3.18), the first enzyme of the biosynthetic pathway that produces the branched chain amino acids valine, leucine, and isoleucine, has been identified as the target site of sulfonylurea (Chaleff and Mauvais 1984), imidazolinone (Shaner et al. 1984), and triazolopyrimidine herbicides (Gerwick et al. 1987). Most corn inbreds and hybrids are tolerant to primisulfuron but sensitive to many structurally related sulfonylureas. Natural plant tolerance to sulfonylurea herbicides has in all reported cases been attributed to the ability to detoxify the herbicide molecule (Sweetser et al. 1982; Hutchison et al. 1984; Neighbors and Privalle 1990). This suggested that the observed primisulfuron tolerance of corn was probably due to a specific metabolic capacity rather than to a herbicide-insensitive form of AHAS. In order to clarify this point, we measured the inhibition of AHAS enzyme activity by primisulfuron in crude extracts from the tolerant inbred 4C0, the sensitive inbred 4N5, and their hybrid. The finding, in these experiments, that AHAS activity from the three lines was equally sensitive clearly excludes the possibility that a herbicide-insensitive form of AHAS is the basis for primisulfuron tolerance in inbred 4C0.

In line with the assumption that differential metabolism could account for the selective tolerance to primisulfuron but not other sulfonylureas, we determined the rate of disappearance of active primisulfuron from treated corn seedling tissues. A clear difference was observed between the tolerant and the sensitive inbred. Primisulfuron metabolism was remarkably faster in the tolerant inbred 4C0 than in 4N5. Consistent with its phenotypic behavior in planta and in the root growth assays. the F_1 hybrid showed the same rate of primisulfuron metabolism as the tolerant inbred, 4C0. Primisulfuron was converted into several metabolites that lack herbicidal activity (Neighbors and Privalle 1990; Fonné-Pfister et al. 1990). These results show that the ability to metabolize primisulfuron correlates with tolerance, and that this ability is heritable as a dominant trait.

The results of our genetic and biochemical analyses indicate that primisulfuron tolerance in corn is based on a capacity to rapidly metabolize the herbicide. This finding is consistent with previous observations regarding the natural tolerance of certain crop or weed species to sulfonylurea herbicides (Sweetser et al. 1982; Hutchison et al. 1984; Neighbors and Privalle 1990). In contrast, mutants selected for resistance to sulfonylurea or imidazolinone herbicides have been found to possess a herbicide-insensitive form of AHAS (Chaleff and Mauvais 1984; Anderson 1986; Haughn and Somerville 1986; Armour et al. 1989). Amplification of an AHAS allele (Armour et al. 1990) indicated yet another possible mechanism. Sebastian and Chaleff (1987) reported soybean mutants whose tolerance to chlorsulfuron behaved as a recessive trait in genetic tests. Our finding of herbicide-sensitive AHAS enzyme activity in the tolerant as well as the sensitive corn inbred clearly disproves the possibility that a herbicide-insensitive form of AHAS was the basis of primisulfuron tolerance in inbred 4C0. The genetic analysis strongly suggested that primisulfuron tolerance was based on a single dominant nuclear gene. Inbred 4N5 contains a nonfunctional allele of this gene in a homozygous state rendering it sensitive to primisulfuron. Further analysis of primisulfuron metabolism in barnyard grass (a tolerant weed) suggested the involvement of a cytochrome-P450 mixed function oxygenase in the detoxification of primisulfuron (Neighbors and Privalle 1990). Further evidence in support of this was reported most recently by Fonné-Pfister et al. (1990), who have shown that hydroxylation by an inducible cytochrome P450-dependent monooxygenase enzyme system is the first step in the metabolism of primisulfuron in corn.

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