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Tolerance to Imidazolinone Herbicides in Wheat

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ABSTRACT

An imidazolinone-tolerant wheat (*Triticum aestivum* L. em Thell) mutant in the winter wheat cultivar Fidel has been identified and characterized. The mutant was isolated from a population derived through seed mutagenesis of the variety with an aqueous solution containing sodium azide. Imidazolinone-tolerant wheat seedlings were selected from the M_2 generation of the population in the presence of imazethapyr herbicide and identified as herbicide-insensitive individuals. The trait is inherited as a single semidominant gene and confers high levels of tolerance to imazethapyr. Acetohydroxyacid synthase activity in extracts from imidazolinone-tolerant plants was less inhibited by imazethapyr than the enzyme from the wild type. The herbicide-tolerant plants have a completely normal phenotype and display no negative effects on growth and yield in either the absence or presence of imazethapyr.

The imidazolinone class of herbicides has been demonstrated to have a broad spectrum of weed control activity, flexibility in timing of application, low usage rates, and low mammalian toxicity. These herbicides inhibit the enzymic activity of AHAS[2] (EC 4.1.3.18) (15), the first enzyme in the pathway for the synthesis of the branched chain amino acids valine, leucine, and isoleucine. This same enzyme has been shown to be the site of action for the sulfonylurea and triazolopyrimidine herbicides (4, 19). The basis for crop selectivity of the imidazolinones has been shown to be the result of a difference in the nature or rate of metabolism of the herbicide (2, 3, 16). The in vitro AHAS activity of species with natural tolerance to the imidazolinones is sensitive to inhibition by the imidazolinones (17).

Development of a crop variety with AHAS activity insensitive to inhibition by the imidazolinones greatly increases the options for weed control in that crop (10). In such a crop, any of the imidazolinone herbicides could be used without concern about phytotoxicity, and the choice of herbicide could be made independently of concerns about crop selectivity. Development of such a tolerant crop could enable the use of more effective, safer, and more cost-effective weed control options than are currently available. Several plant AHAS mutants have been isolated with similar objectives, which include corn (1), canola (20), soybean (14), and tobacco (5). Several AHAS mutants have also been isolated in *Arabidopsis* (6, 7). These mutants exhibit tolerance to only one or to more than one class of AHAS inhibitors. We attempted to find imidazolinone-tolerant wheat (*Triticum aestivum* L. em Thell) through seed mutagenesis and selection with an M_2 seedling screen.

MATERIALS AND METHODS

Seed Mutagenesis

Five thousand seeds of the French winter wheat (*Triticum aestivum* L. em Thell cv Fidel) were mutagenized by the procedure of Kueh and Bright (9). Wheat seeds were soaked in water for 18 h at 5°C. Air was then bubbled through the seeds for 6 h at 20°C. Following imbibition, seeds were soaked in 1 mM sodium azide (pH 3) for 2 h. The seeds were rinsed for 30 min, dried on paper towels, and planted in the field. The plants arising from these seeds were M_1 generation plants. M_2 seeds were harvested from these plants at maturity.

Screening Procedure

M_2 seeds were surface disinfected in 70% ethanol for 30 s. This was followed by a disinfection in a solution of 2.6% sodium hypochlorite for 30 min under vacuum with gentle agitation. Two drops of the nonionic surfactant Tween 20 were used per 100 mL of the sodium hypochlorite solution to aid the disinfestation procedure. Subsequently, seeds were rinsed three times in sterile distilled water and placed into sterile plastic 10- × 1.5-cm Petri dishes at 250 seeds/dish. Twenty-five milliliters of 1 M imazethapyr was placed in each dish. After 3 d in the imazethapyr solution, the seeds were drained and blotted dry. The seeds were planted at a 1- to 2-cm depth in 15- × 20-cm peat flats containing Metromix 350 (W.R. Grace Co.) at the rate of 1000 seeds/flat. These flats were watered and then sprayed immediately with imazethapyr applied at the rate of 300 $g \cdot ha^{-1}$ in a spray volume of 950 $L \cdot ha^{-1}$ with a belt sprayer. Seeds of Fidel that had not undergone mutagenesis were included, with and without the herbicide treatments, to provide controls that monitored the effectiveness of the selection procedure. After 4 weeks, emerged seedlings were evaluated for herbicide tolerance. Putative tolerant plants were transplanted into 19-cm diameter peat pots filled with Metromix 350 and grown to maturity. Seeds from these putative tolerant plants were subjected to the same selection procedure for confirmation that the selected phenotype was transmitted to the progeny.

Inheritance and Allelism Studies

Homogeneous nonsegregating tolerant progenies for each of the confirmed mutations were obtained after one or two

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[2] Abbreviation: AHAS, acetohydroxyacid synthase.

selfing generations. Homogeneity was established by growing progeny from individual plants in flats and spraying the emerged seedlings at the two-leaf stage with imazethapyr. Imazethapyr was applied postemergence at 62.5 $g \cdot ha^{-1}$ in a spray volume of 950 $L \cdot ha^{-1}$ with a belt sprayer. The surfactant Tween 20 was included in the spray solutions at 0.25% (v/v). Under these conditions, tolerant individuals were relatively unaffected, and seedlings from the cv Fidel (unselected) were uniformly susceptible.

After homogeneous tolerant lines were obtained, all possible crosses and their reciprocals were made between the following five lines: the progenitor cv Fidel and four tolerant selections. The tolerant selections were named FS1, FS2, FS3, and FS4, i.e. Fidel selection No. 1, Fidel selection No. 2, etc. The hybrids resulting from the crosses were then both selfed and backcrossed again to Fidel. Parental lines, F_1 hybrids, selfs of the hybrids, and test crosses were planted into flats and sprayed with imazethapyr at 200 $g \cdot ha^{-1}$, by the method detailed above, to evaluate the inheritance and allelism patterns for the selected mutations.

Greenhouse Characterization of Herbicide Tolerance

M_4 seeds of the mutant lines that had been progeny tested and established to be homogeneous for tolerance were pooled for each of the four selections. Seeds from the four selections and from Fidel were planted into 15-cm diameter pots filled with Metromix 350 at five seeds per pot and thinned to three plants per pot after emergence. The plants were sprayed postemergence at 10 d after planting with the following herbicides at the rates listed: imazethapyr, at 20, 40, 80, 160, and 320 $g \cdot ha^{-1}$; imazaquin, at 40, 80, 160, 320, and 640 $g \cdot ha^{-1}$; imazapyr, at 3, 6, 12, 24, and 48 $g \cdot ha^{-1}$; and sulfometuron methyl, at 5, 10, 20, 40, and 80 $g \cdot ha^{-1}$. Three replications of each herbicide-rate combination were used for each genotype in a completely randomized design. Herbicide treatments were applied with a belt sprayer in a spray volume of 400 $L \cdot ha^{-1}$. Tween 20 was added at 0.25% (v/v) to all spray solutions. Plant heights were measured using the extended leaf method before spraying and at 1-week intervals for the following 4 weeks. Plants surviving the herbicide treatment were scored as resistant, and plants killed by the herbicide treatment were scored as susceptible.

Table I. *Observed Segregation Pattern for the Imidazolinone Tolerance Present in Selections FS1 to FS4*

Seedlings from F_2 populations were treated postemergence with a rate of 200 $g \cdot ha^{-1}$ of imazethapyr. Tolerant (T) plants survived the herbicide treatment, whereas susceptible (S) plants were killed by the treatment.

Parents of F_2 Population	F_2 Segregation, Observed (T:S)	F_2 Segregation, Expected (T:S)	χ^2 Fit of Observed to Expected
FS1 × Fidel	407:155	421.5:140.5	2.00, NS[a]
FS2 × Fidel	514:164	510.8:170.3	0.31, NS
FS3 × Fidel	419:138	417.8:139.3	0.01, NS
FS4 × Fidel	424:159	437.3:147.8	1.61, NS

[a] The observed segregation is not significantly (ns) different, by a χ^2 test at the 5% level, from that expected for a 3:1 tolerance:sensitive ratio.

Table II. *Expression of Tolerance in the F_2 Populations Produced by Selfing Hybrids Made between the Four Imidazolinone-Tolerant Selections, FS1 to FS4*

Seedlings from F_2 populations were treated postemergence with a rate of 200 $g \cdot ha^{-1}$ of imazethapyr. Tolerant (T) plants survived the herbicide treatment, whereas susceptible (S) plants were killed by the treatment.

First Parent	Second Parent		
	FS2	FS3	FS4
FS1	513:4	553:1	557:4
FS2		564:1	580:2
FS3			580:0

Enzyme Assay for AHAS Activity

The procedures used for the in vitro assays of AHAS activity have been published (18). Acetolactate produced by the enzyme was acid converted to acetoin, which was measured by the Westerfeld reaction (18). Either leaf blades or lower portions of shoots were used as a source of plant tissue for the assay. The assays were performed at least two times with a minimum of two replications of each assay. All chemicals were purchased from Sigma Chemical Co.

Field Studies

A field trial was conducted in 1989 to 1990 on the American Cyanamid Agricultural Research site located near Princeton, NJ. The trial examined herbicide tolerance and grain yield for susceptible and imidazolinone-tolerant wheat. A split plot design was used, with herbicide-rate combinations as main plots and genotypes as the split plots. The plot size for each experimental unit was 2.5 m long and 1.24 m wide. Treatments were applied postemergence in the spring in a spray volume of 200 $L \cdot ha^{-1}$ with a backpack sprayer. X-77 was used as a nonionic surfactant at a rate of 0.25% (v/v). The treatments were applied when the plants were approximately 30 to 35 cm tall. Plant height data was taken at 3 and 6 weeks after treatment. Grain yields were measured at maturity, approximately 10 weeks after herbicide treatment.

RESULTS

Approximately 120,000 M_2 seeds were harvested from 5,000 M_1 seeds planted in the field. The germination percentage for the harvested M_2 seeds was close to 100%. The M_2 seeds were screened for herbicide-tolerant individuals as described in "Materials and Methods." It was determined during the development of the screen that, by using both a seed-soaking procedure and a preemergent spray, the frequency of escapes could be reduced substantially. In addition, the two-step screening approach allows for weaker mutations to emerge and grow more readily than would be permitted by increasing herbicide concentrations at either of the two selection steps individually. The exact concentrations of herbicide used for the selection process are specific to the conditions used and will vary with the genotype, growth media, and growth conditions used for selection.

Four wheat plants with putative tolerance to imazethapyr

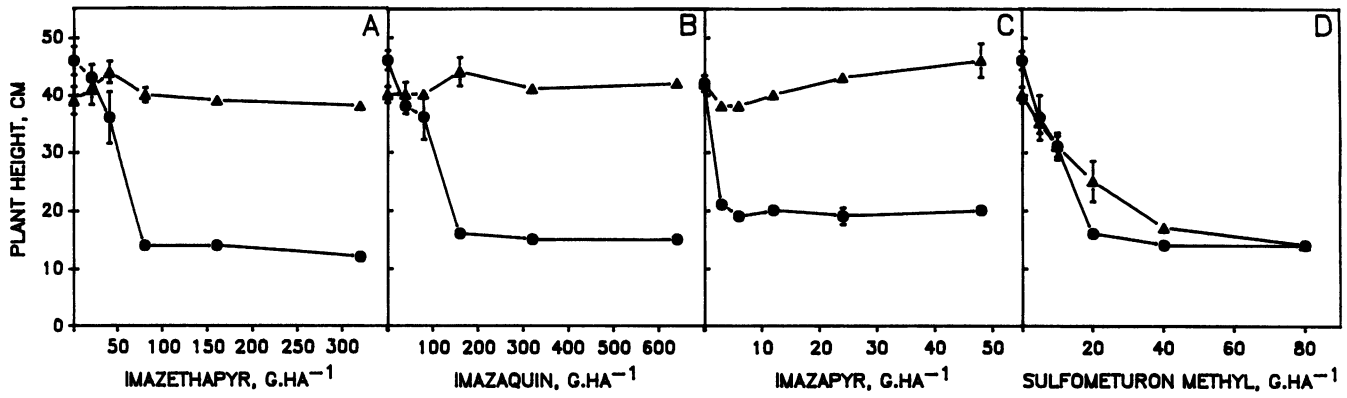

Figure 1. Mean plant heights of Fidel (●) and FS4 (▲) wheat measured 4 weeks after treatment with herbicides in a greenhouse test. The herbicides were applied postemergence and included imazethapyr (A), imazaquin (B), imazapyr (C), and sulfometuron methyl (D). The values are the average of three numbers. Error is indicated when it is larger than the symbol.

were identified by this procedure and named FS1 (i.e. Fidel selection No. 1), FS2, FS3, and FS4. The selections were transplanted to pots and grown to maturity in the absence of additional herbicide. M_3 seeds from each of the four plants selected were screened for imidazolinone tolerance. The results showed that the herbicide tolerance trait was transmitted to the progeny of the selections. Selected M_3 generations of FS1, FS2, and FS4 were apparently homozygous for the tolerance trait because the progeny from these selections did not segregate for tolerance. FS3 was apparently heterozygous for tolerance because it required an additional generation of selfing to identify progenies nonsegregating for the tolerance trait. In addition to the selection for tolerance, some visual selection was made to eliminate undesirable growth characteristics (shorter stature, poor vigor, etc.) that were present as a result of the mutagenesis procedure.

The four selections and the parental cv Fidel were crossed in all possible combinations, including reciprocal crosses. F_1 hybrids between the selections and Fidel were treated postemergence at 50 to 250 g·ha^{-1} of imazethapyr. Hybrids were slightly stunted at the highest rate used but survived and grew out of the damage. F_1 hybrids of crosses among the four selections were unaffected by all rates of imazethapyr used. No segregation was noted among the hybrids for tolerance; all were uniformly tolerant. Conversely, Fidel (without the tolerance trait) was severely damaged at 50 and 100 g·ha^{-1} and killed at rates of 150 g·ha^{-1} of imazethapyr and higher in the same test. These data indicate that the tolerant parents were homozygous for tolerance and that the tolerance is inherited as a semidominant trait. Reciprocal crosses provided similar results, and no indication for maternal inheritance of the trait was noted.

F_2 progenies from the crosses between Fidel and each of the tolerant selections segregated in a 3:1 tolerant:susceptible ratio (Table I), indicating that tolerance is inherited as a single semidominant gene for each of the selections. The segregation within the progenies was evaluated by a postemergence treatment of seedlings at 200 g·ha^{-1} of imazethapyr, a rate at which heterozygous individuals are relatively unaffected and susceptible individuals are killed.

The intercrosses between the selections were also selfed and the resulting progenies screened for segregation of the tolerance trait under the same conditions. If two independently segregating tolerance genes were present, one of 16 of the F_2 plants derived from the intercross would be susceptible to treatment by imidazolinone herbicide. The number of

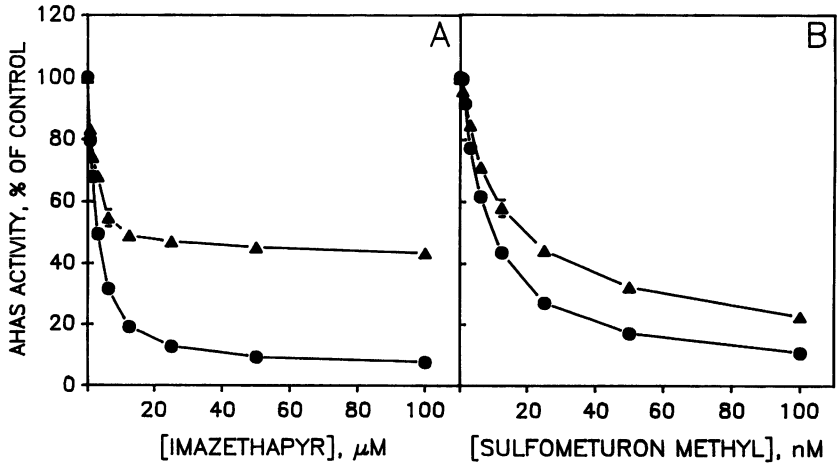

Figure 2. Inhibition of AHAS activity from Fidel (●) and FS4 (▲) by the AHAS-inhibiting herbicides imazethapyr (A) and sulfometuron methyl (B). AHAS was extracted from seedling tissue and assayed under standard conditions. The values are the average of two numbers. Error is indicated when it is larger than the symbol.

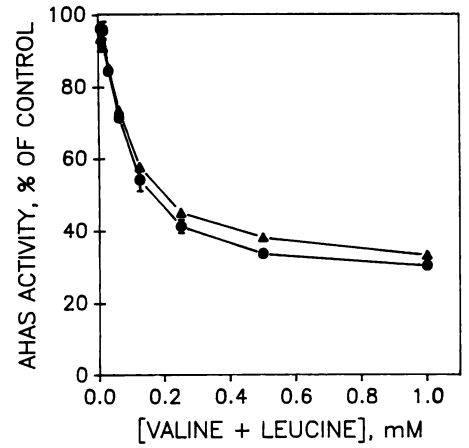

Figure 3. Inhibition of AHAS activity from Fidel (●) and FS4 (▲) by valine and leucine. AHAS was extracted from seedling tissue and assayed under standard conditions. The concentrations presented here represent the concentration of each amino acid. The values are the average of two numbers. Error is indicated when larger than the symbol.

susceptible plants observed did not approach one-sixteenth of the total number tested for any of the crosses (Table II); therefore, it appears that all of the tolerance mutations are either alleles at the same locus or at very closely linked loci. The few susceptible plants observed are believed to have resulted from remnant deleterious effects caused by the mutagenesis procedure and as such were unrelated to segregation of the tolerance gene. It is possible that all four selections are derived from the same mutational event, given the relatively small population size screened and the similarity of phenotypic characteristics that were noted for each of the four selections. For this reason, tolerance data from only one selection will be given to represent the response of all four selections in the rest of the paper.

The FS4 selection expresses excellent tolerance to postemergence applications of imazethapyr, the selection agent, in greenhouse trials (Fig. 1A). The commercial use rate of imazethapyr on soybeans is 70 $g \cdot ha^{-1}$; the imidazolinone tolerance gene provided at least a 4-fold margin of safety to that rate in this test. The FS4 tolerance gene also confers tolerance to the imidazolinones imazaquin and imazapyr (Fig. 1, B and C). Alternatively, only marginal tolerance to the sulfonylurea herbicide sulfometuron methyl is present in the selections (Fig. 1D). Similar results have been observed in greenhouse trials in which tolerance for preemergence applications of these herbicides were examined (data not shown).

As for the whole plant tolerance data, the AHAS activity profiles for the four selections FS1 to FS4 were all similar (data not shown). The sensitivity of the AHAS enzyme activity from Fidel and the tolerant selection FS4 to inhibition by the herbicides imazethapyr, imazaquin, imazapyr, and sulfometuron methyl was checked by in vitro assays with extracts from seedlings of the two genotypes. Much of the AHAS activity from the tolerant line FS4 was insensitive to imazethapyr when compared with the inhibition curve for the normal enzyme from Fidel (Fig. 2A). The AHAS activity profiles for imazaquin and imazapyr are similar to that for imazethapyr (data not shown). The AHAS activity in extracts of FS4 also displayed moderate levels of insensitivity to sulfometuron methyl (Fig. 2B). AHAS activity from all selections exhibited in vitro feedback regulation by valine and leucine that is indistinguishable from feedback sensitivity of the AHAS activity from susceptible Fidel (Fig. 3).

Susceptible and tolerant wheat varieties were planted in field trials in the fall of 1989. Fidel is a winter wheat variety adapted to France and, therefore, not well adapted to the Princeton, NJ, environment. The intent of the field trial was to evaluate field tolerance to imazethapyr rather than to assess agronomic potential. Fidel and FS4 were treated in the spring of 1990 with imazethapyr at 200 $g \cdot ha^{-1}$. Plant heights and grain yields of Fidel were decreased when treated with imazethapyr as compared to the untreated controls (Table III). Little or no effect of imazethapyr treatment was observed for FS4. Both Fidel and FS4 were sensitive to treatment with the sulfonylurea herbicide nicosulfuron, exhibiting reductions in both plant height and grain yield.

DISCUSSION

Screening of progeny from seeds treated with a mutagen has been demonstrated to be an effective method for identifying herbicide-tolerant mutations in plants, both for recessive mutations (8, 12, 13) and for dominant mutations (6, 7, 14). Herbicides provide a potent and repeatable selection agent for plants. By using seedlings to screen for mutations, we may avoid potential pitfalls associated with in vitro selection procedures, such as the need for differentiated tissues for expression of the trait.

The characteristics of the imidazolinone-tolerant wheat described in this study are similar in many respects to other imidazolinone-tolerant and sulfonylurea-tolerant plant selections previously reported in the literature (1, 5, 6, 11, 14, 20). The tolerance trait is inherited as a single semidominant gene. When in the homozygous state, the mutation provides strong tolerance to the imidazolinone herbicides tested but little or

Table III. *Plant Heights and Grain Yields Measured on Fidel and FS4 Winter Wheat Treated Postemergence with Imazethapyr and Nicosulfuron Herbicides in a 1989 to 1990 Field Trial Conducted at a Site Near Princeton, NJ.*

Genotype	Herbicide Treatment	Plant Height		Grain Yield
		3 WAT[a]	6 WAT	
		cm		$g \cdot m^2$
Fidel	Untreated	84	93	180
	Imazethapyr, 200 $g \cdot ha^{-1}$	50	58	22
	Nicosulfuron, 40 $g \cdot ha^{-1}$	58	59	2
FS4	Untreated	79	89	174
	Imazethapyr, 200 $g \cdot ha^{-1}$	78	91	154
	Nicosulfuron, 40 $g \cdot ha^{-1}$	54	59	2
LSD 0.05		6	7	34

[a] WAT, Weeks after treatment.

no tolerance for the sulfonylurea herbicides sulfometuron methyl and nicosulfuron. These results are consistent with the notion that imidazolinones and sulfonylureas do not have the same binding site on the enzyme (6, 7, 11, 20).

The imidazolinone tolerance in wheat reported here is conferred through AHAS activity that is insensitive to inhibition by the imidazolinone herbicides (Fig. 2A). However, the smaller changes in insensitivity of AHAS activity to sulfometuron methyl noted in Figure 2B did not confer significant levels of tolerance at the whole plant level (Fig. 1D). Because only a portion of the AHAS activity in the tolerant wheat plants was insensitive to inhibition by imidazolinones, it is likely that two or more genes for the AHAS enzyme exist in wheat, only one of which has been altered. Findings of multiple loci for AHAS in plants has been observed in tobacco (5) and canola (20).

Inhibition of AHAS activity by the feedback inhibitors valine and leucine was similar for the enzyme from the normal and imidazolinone-resistant wheat seedlings. These results are consistent with the finding of unaltered feedback regulation of AHAS from imidazolinone-resistant or sulfonylurea-resistant mutant lines (ref. 11 and references cited therein). These results reiterate our earlier conclusion that the herbicide's binding sites and the feedback inhibitors' binding sites are distinct (11).

The incorporation of this tolerance gene in wheat could expand the opportunities for weed control in the crop. Because the gene confers tolerance through an altered site of action, the use of imidazolinone herbicides with desirable weed control and environmental safety characteristics that were previously nonselective in wheat is enabled. The field trials that were conducted indicate that level of tolerance is adequate under field conditions, and no deleterious effects of the gene were observed either in the absence or presence of imazethapyr.

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