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A Mutation Causing Imidazolinone Resistance Maps to the *Csr1* Locus of *Arabidopsis thaliana*[1]

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ABSTRACT

A mutant of *Arabidopsis thaliana*, two hundred times more resistant to the imidazolinone herbicide imazapyr than wild-type plants, was isolated by direct selection of seedlings from a mutagenized population. Genetic analysis showed that resistance is due to a single dominant nuclear mutation that could not be separated by recombination from a mutation in the *CSR1* gene encoding acetohydroxy acid synthase. Acetohydroxy acid synthase activity in extracts isolated from the mutant was 1000-fold more resistant to inhibition by imazapyr than that of the wild type. The resistant enzyme activity cosegregated with whole plant resistance. These data strongly suggest that the mutation is an allele of *CSR1* encoding an imazapyr-resistant AHAS.

AHAS[2] (EC 4.1.3.18), also known as acetolactate synthase, the first enzymatic step common to the biosynthesis of the three branched-chain amino acids, is the site of action of the structurally diverse classes of herbicides: the sulfonylureas and imidazolinones (13, 18). Single dominant mutations resulting in resistance to sulfonylurea compounds in microorganisms (6, 23) and plants (2, 3, 7) have been shown to be due to single base pair substitutions in genes encoding the AHAS catalytic subunit (10, 14, 23).

Although the molecular basis for imidazolinone-resistance has not yet been reported, several lines of evidence suggest that mutations causing such a phenotype will also lie within the AHAS gene. First, enzyme kinetics data have shown that the imidazolinones, like the sulfonylureas, inhibit AHAS activity by binding noncompetitively to a common site on the enzyme (17). Second, a number of variant cell lines of *Datura innoxia* resistant to sulfonylurea herbicides showed cross-resistance to imidazolinone herbicides (16). Finally, mutants of *Chlamydomonas reinhardtii* resistant to imidazolinones, chlorsulfuron or both map to the same genetic locus (22).

We report here the isolation and characterization of an imazapyr-resistant mutant of *Arabidopsis thaliana* and show that resistance is due to a dominant mutation which maps to the AHAS locus. The recent cloning and characterization of the *A. thaliana* AHAS gene (10, 15) should facilitate molecular analysis of imidazolinone-resistance in this mutant.

MATERIALS AND METHODS

Reagents

Chlorsulfuron was obtained from Chem Service Inc., West Chester, PA. Imazapyr (AC252,925) was generously provided by D. L. Shaner, American Cyanamid Co., Princeton, N.J. This compound is similar to AC243,997 described by Shaner *et al.* (18). Herbicides were stored at −20°C as stock solutions at the following concentrations: chlorsulfuron, 1 mg mL^{-1} in 10 mM potassium phosphate (pH 7.5); imazapyr, 50 mM in water.

Lines, Growth Conditions, and Genetic Methods

Unless otherwise stated, all lines of *Arabidopsis thaliana* used in this study were derived from the Columbia ecotype. Chlorsulfuron-resistant line GH50 (*csr1*) and line MSU53 (*gl1*) have been described previously (7). Chromosome three linkage marker stocks, lines MSU22 (*hy2, gl1, tt5, er*) and MSU26 (*gl1, cer7, er*) (Landsberg ecotype), were obtained from M. Koornneef (11, 12). Standard genetic nomenclature for *Arabidopsis*[3] is used throughout the manuscript.

The following methods have been described previously: the growth and handling of plants in soil and on defined minimal agar under sterile conditions (7); ethyl methane sulfonate mutagenesis (7); cross-fertilization, handling of seed (19), and selection of herbicide-resistant mutants in *A. thaliana* (8).

Herbicide resistance was scored as follows. Seeds were surface-sterilized and sown on minimal agar plates. After 5 to

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[2] Abbreviations: AHAS, acetohydroxy acid synthase; *imr*, symbol for a gene encoding imazapyr resistance; chlorsulfuron, (2-chloro-*N*-[(4-methoxy-6-methyl-1,3,5-triazin-2-1)aminocarbonyl] benzenesulfonamide); imazapyr, isopropylamine salt of 2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl) nicotinic acid.

[3] The genetic nomenclature used here follows the recommendations developed by an *ad hoc* committee at the Third International *Arabidopsis* Meeting, Michigan State University, April 1987, as follows: (a) genotypes are italicized and typically consist of three letters; (b) where the gene symbol is followed by a number, to distinguish between different genes with the same three letter designation, it is not separated by a hyphen; (c) the genotype of the wild type is capitalized and the genotype of mutant alleles is lower case; (d) phenotypes are designated by the gene symbol that is not italicized but has the first letter capitalized; (e) when more than one allele is known for a gene, alleles are specified by a hyphenated number; (f) dominance relationships are not indicated in genotypic designations.

9 d (first pair of leaves visible), seedlings were transferred to minimal agar containing an appropriate concentration of herbicide (5 μM imazapyr and 0.1 μM chlorsulfuron, unless otherwise stated). Plants showing growth of root or shoot 4 to 7 d after transfer were scored as resistant. If it was necessary to score for resistance to an additional herbicide, resistant progeny were transferred to new plates containing the second herbicide and scored after an additional 4 to 7 d.

Enzyme Assays

Crude extracts of *A. thaliana* leaf tissue were prepared by harvesting approximately 1 g of leaves from 3 to 4-week-old plants (plants just beginning to flower) and homogenizing the tissue in 2 mL of cold extraction buffer containing 100 mM potassium phosphate buffer (pH 7.5), 0.5 mM $MgCl_2$, and 1 mM sodium pyruvate. Debris was pelleted for 15 min at 4°C and 27,000*g* and the supernatant assayed immediately. When extracts were prepared from individual plants, 0.05 to 0.15 g of leaf tissue was homogenized in 300 μL of extraction buffer and debris was removed by centrifugation for 10 min, at 17,000*g* and 4°C.

AHAS activity was assayed using the procedure of Chaleff and Mauvais (2). A reaction volume of 500 μL included 150 μL of extract, 65 mM potassium phosphate buffer (pH 7.5), 40 mM sodium pyruvate, 10 mM $MgCl_2$ 250 μM thiamine pyrophosphate, 23 μM flavin-adenine dinucleotide, and the indicated concentration of herbicide. The reaction mixture was incubated at 30°C for 90 min and stopped by addition of 250 mL of 6 N H_2SO_4. Acetolactate was converted to acetoin by incubation of the reaction mixture at 56°C for 10 min. The pH of the solution was adjusted to 7.0 with NaOH and the precipitate removed by centrifugation for 5 min at 4°C and 17,000*g*. The acetoin concentration in 850 μL of the supernatant was determined indirectly (21) by the addition of 150 μL of 0.5% creatine and 150 μL of 5.0% 1-naphthol (made immediately beforehand in 2.5 N NaOH), and measurement of OD^{530} after incubation of the solution at 56°C for 20 min. Protein concentrations in the extracts were measured by the method of Bradford (1).

RESULTS

Isolation of Imazapyr-Resistant Mutants

Wild type *Arabidopsis thaliana* seedlings were unable to grow on minimal agar medium containing concentrations of the herbicide imazapyr higher than 0.05 μM, unless the medium was supplemented with isoleucine and valine (1 mM each). Three putative imazapyr-resistant seedlings (GH90–GH92) were identified by germinating 150,000 M2 seeds (progeny of ethyl methane sulfonate-mutagenized plants) on minimal agar plates (6000–8000 seeds/plate) containing 10 μM imazapyr. All three of the plants selected were derived from the same subpopulation of 25,000 M1 seedlings. Resistant plants were transferred to soil and grown to maturity without further selection. Fifty-nine of the 61 progeny of line GH90 tested grew on 5 μM imazapyr indicating that imazapyr resistance in this line was a heritable trait and that the original isolate was homozygous for the mutation(s) causing imazapyr-resistance. Line GH90 was confirmed to be homozygous for resistance to imazapyr both by analysis of seed from subsequent generations and by genetic analysis (see below). Isolates GH91 and GH92 produced both sensitive and resistant progeny suggesting that they were heterozygous for a dominant allele conferring imazapyr resistance. Preliminary analysis of these lines suggested that they are similar to line GH90 genetically and phenotypically. For this reason only the latter was analyzed in detail.

Level of Resistance

The degree of imazapyr resistance of line GH90 was determined by transferring 5-d-old (cotyledon stage) seedlings to minimal agar plates containing one of several different concentrations of imazapyr. The mean fresh weight of plants 15 d after transfer is shown in Figure 1. Wild-type plants were able to grow to maturity on 0.02 μM imazapyr, grew slowly on 0.05 μM imazapyr, and were killed by concentrations of 0.2 μM imazapyr and higher. By contrast, line GH90 was unaffected by concentrations of imazapyr as high as 2 μM and could grow to maturity at 5 μM imazapyr; plants grown at 5 μM, however, often had narrow leaves and were darkly pigmented. In the absence of herbicide, plants of line GH90 were slightly chlorotic and smaller than wild-type plants of the same age (Fig. 1). The loss of vigor and herbicide resistance, however, did not segregate together during subsequent genetic crosses (data not shown) indicating that these phenotypes were the result of different mutational events.

Genetic Analysis of GH90

The genetic basis for imazapyr resistance was investigated by outcrossing to herbicide-sensitive line MSU22 which carries genetic markers for chromosome three. All the F1 progeny

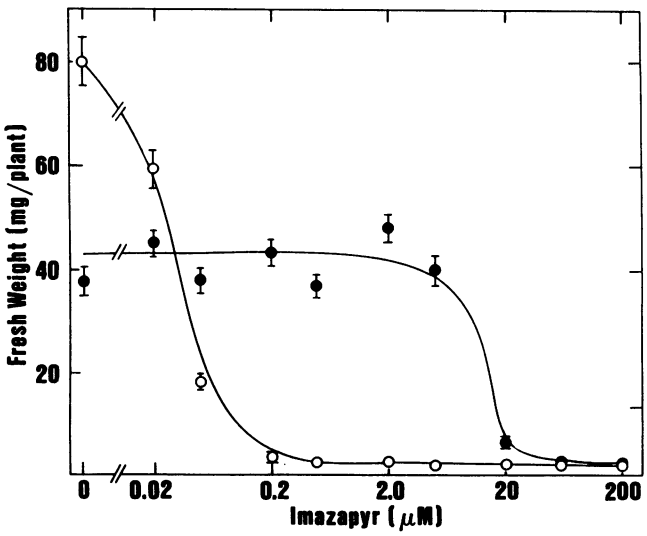

Figure 1. Growth of wild type and mutant line GH90 on imazapyr. For both wild type (○) and line GH90 (●), the average fresh weight of 30 individual plants measured 15 d after transfer to herbicide is given. The vertical bars at each data point represent the standard error of the mean.

were resistant to 5 μM imazapyr and resistance segregated 3:1 in the F_2 progeny (Table I; $\chi^2 = 0.6$, $P \geq 0.9$). Thus, the imazapyr resistance is the result of a single dominant nuclear mutation in a gene we tentatively designate *Imr*.

Imazapyr-resistant F2 progeny from the MSU22 × GH90 cross were transferred to soil, grown to maturity, and scored for the genetic markers *hy2*, *gl1*, and *tt5*. Results of this analysis (Table I) demonstrate linkage of *imr* to both *gl1* and *tt5*, but not *hy2*. It was possible to use the results in Table I to estimate the number of plants in each of the herbicide sensitive phenotypic classes by assuming that the recombinant classes *TT5/—*, *IMR/IMR* and *GL1/—*, *IMR/IMR* were equal to the resistant reciprocal classes *gl1/gl1*, *imr/—*; and *tt5/tt5*, *imr/—*, respectively. With these data, approximate map distances were calculated (Table II) placing *imr* on chromosome three, between *GL1* and *TT5* loci close to the *CSR1* locus (Table II; 4, 7).

Imidazolinones are known to inhibit AHAS (18). Therefore, it was conceivable that *imr* is an allele of *CSR1*, the gene encoding AHAS in *A. thaliana*. To determine more accurately the map distance between *imr* and *CSR1*, lines GH90 (*imr*) and chlorsulfuron-resistant line GH50 (*csr1*) were crossed. As expected, all of the eight F_1 progeny tested were resistant to both chlorsulfuron and imazapyr. The F_1 progeny were then either crossed to MSU8 (test cross) or allowed to self-fertilize to produce F_2. The progeny from these crosses were initially scored for resistance to either chlorsulfuron or to imazapyr (Table III). As expected, progeny segregated 1 resistant:1 sensitive (test cross progeny) or 3 resistant:1 sensitive (F_2 progeny). The resistant plants from each cross were tested for cross-resistance to the second herbicide. For the test cross progeny, all 79 plants resistant to imazapyr were sensitive to chlorsulfuron and all 82 plants resistant to chlorsulfuron were sensitive to imazapyr. Approximately two-thirds of the 168 F_2 progeny (Table III) initially selected for resistance to imazapyr were resistant to chlorsulfuron (106:62, resistant:sensitive). Similarly, approximately two-thirds of the 167 chlorsulfuron-resistant progeny were found to be resistant to imazapyr (98:69, resistant:sensitive). These segregation data indicate that *imr* and *csr1* are very closely linked. The most convincing evidence of this was the fact that of 353 test cross progeny examined (Table III, MSU8 × [GH50 × GH90]), none showed the recombinant phenotype (resistance to both herbicides).

Inhibition of AHAS Activity by Imazapyr

As shown above, *imr* maps closely and may be allelic to the *CSR1* locus. Because *CSR1* encodes AHAS in *A. thaliana*, and AHAS is known to be strongly inhibited by imidazolinone herbicides (18), resistance in line GH90 could be due to synthesis of an imazapyr-resistant AHAS, overproduction of AHAS, or inactivation of the herbicide. To distinguish between these possibilities, AHAS activities in crude extracts derived from wild type, line GH90, or wild type × GH90 F_1 plants were measured in the presence of several different concentrations of imazapyr. In the absence of herbicide, the specific activity of AHAS was similar in all three lines (Fig. 2) suggesting that resistance is not due to overproduction of AHAS activity. AHAS activity in extracts from wild-type plants was almost completely inhibited by imazapyr concentrations of 20 μM and higher (Fig. 2A). In contrast, AHAS activity in GH90 extracts was inhibited only 20% by 500 μM imazapyr (Fig. 2A). Fifty percent of the AHAS activity in extracts from the wild-type-GH90 F_1 heterozygote was highly resistant to inhibition to imazapyr. These results suggest that imazapyr resistance is due to a resistant enzyme activity, not to an inactivation of the herbicide.

Cosegregation of Whole-Plant Resistance with Resistant AHAS Activity

If the imazapyr-resistant AHAS activity described above is the cause of whole-plant resistance in GH90, the two phenotypes should segregate as a single genetic locus. To test this, F_2 progeny of the cross MSU22 × GH90 were individually assayed for AHAS activity in both the presence and absence of 50 μM imazapyr. At this concentration of imazapyr, resistant and sensitive individuals could easily be distinguished

Table I. *Genetic Segregation and Linkage Analysis of imazapyr Resistance in Line GH90*

Cross	Number of Plants				χ^2
	Resistant		Sensitive		
	Observed	Expected[a]	Observed	Expected[a]	
GH90 × MSU22[b] F1	9	NA[c]	0	NA	NA
MSU22 × GH90 F1	11	NA	0	NA	NA
GH90 × MSU22 F2	201	206.25	74	68.75	0.5
MSU22 × GH90 F2	189	183.75	56	59.25	0.6
GL1/___	163	141.75	ND[d]	ND	12.8
gl1/gl1	26	47.25	ND	ND	
TT5/___	169	141.75	ND	ND	20.9
tt5/tt5	20	47.25	ND	ND	
HY2/___	142	141.75	ND	ND	0.002
hy2/hy2	47	47.25	ND	ND	

[a] Expected numbers were calculated assuming 3:1 segregation (independent assortment). [b] MSU22 carries the following markers on chromosome three: *hy2*, *gl1*, and *tt5*. [c] Not applicable. [d] No data.

Table II. *Estimates of Recombination Percentages between imr or csr1-1 and Markers on Chromosome Three*

Recombination percentages and standard deviations were calculated by the method of Suiter *et al.* (20) and converted to map distance in centiMorgans using the Kosambi mapping function (11). Data for *imr* were obtained as described in the text. For crosses involving *csr1*, all markers were scored in the F_2 generation except *csr1* which was scored by testing F_3 progeny of individual F_2 plants for resistance to 0.1 μM chlorsulfuron.

Cross[a]	Markers	Recombination	Map Distance
			cM
MSU22 × GH90	*imr/gl1*	25.3 ± 5.9	27.9 ± 7.9
	imr/tt5	18.7 ± 6.1	19.6 ± 7.1
MSU22 × GH50	*tt5/gl1*	33.8 ± 6.4	41.1 ± 11.8
	tt5/csr1	9.1 ± 7.4	9.1 ± 7.7
	csr1/gl1	28.1 ± 6.8	31.7 ± 9.9
MSU26 × GH50	*cer7/gl1*	36.1 ± 5.3	45.5 ± 11.1
	cer7/csr1	17.3 ± 6.0	18.0 ± 6.8
	csr1/gl1	23.8 ± 5.8	25.9 ± 7.5

[a] Parental markers carried: MSU22 (*hy2*, *gl1*, *tt5*, *er1*); MSU26 (*gl1*, *cer7*, *er1*); GH50 (*csr1*).

Table III. *Segregation and Linkage Analysis of imr and csr1*

F_2 or test cross progeny were scored for resistance to one of two herbicides (0.1 μM chlorsulfuron or 5 μM imazapyr).

Cross	imazapyr		chlorsulfuron	
	Resistant	Sensitive	Resistant	Sensitive
GH50 × GH90 F_2	168	62	167	49
MSU8 × (GH50 × GH90)	79	103	82	89

from one another on the basis of percentage of activity in the absence of herbicide (see Fig. 2A). Whole plant resistance was scored by testing progeny of each F_2 plant for resistance to 5 μM imazapyr. Of 38 F_2 plants analyzed in this way, 31 were resistant and 7 were sensitive for resistance at both the whole-plant and enzyme levels. The cosegregation of imazapyr resistance at the enzyme and whole plant levels suggest that both phenotypes were due to the same mutation.

Resistance of GH90 to Sulfonylurea Herbicides

Line GH90 was tested for resistance to chlorsulfuron, a sulfonylurea herbicide which strongly inhibits AHAS activity in *A. thaliana* (7). Neither wild type nor GH90 could grow on concentrations of chlorsulfuron higher than 3 nM. Similarly, AHAS activity of line GH90 was only slightly more resistant to inhibition by chlorsulfuron than that of wild type (Fig. 2B).

DISCUSSION

We have isolated, by direct selection, a mutant line of *A. thaliana* highly resistant to the imidazolinone herbicide, imazapyr. Resistance is due to a single dominant nuclear mutation (*imr*) that cosegregates with an imazapyr-resistant AHAS activity. These results suggest that AHAS is the only site of action of imidazolinone herbicides, a hypothesis con-

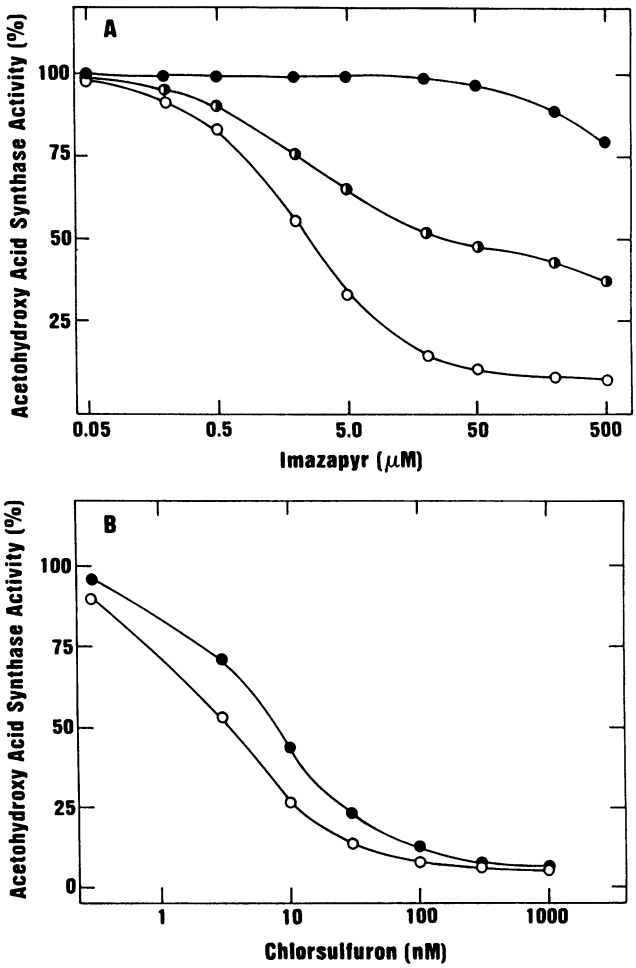

Figure 2. Effect of herbicides on the AHAS activities in extracts from imazapyr-resistant and -sensitive lines of *A. thaliana*. Specific activities (nmol acetoin/mg protein/min) were determined in the presence of the indicated amount of herbicide and are expressed as a percentage of the specific activity obtained in the absence of herbicide. Values given are the averages of results from three independent experiments using extracts from lines GH90 (●), wild-type (○) or two independent experiments using extracts from wild type × GH90 F_1 progeny (◑). The percentage of the specific activities never varied from the mean by more than four percentage points. In the absence of herbicide, the average specific activities and SD from the three experiments were: GH90, 1.74 ± 0.27; wild type, 1.81 ± 0.31; (wild-type × GH90) F_1, 1.52 ± 0.32. A, Effect of imazapyr on AHAS activity; B, effect of chlorsulfuron on AHAS activity.

sistent with previous studies on imidazolinone-resistant mutants in *C. reinhardtii* (22) and *D. innoxia* tissue culture (16).

The *CSR1* gene is the only locus known to encode AHAS in *A. thaliana* and an allele, *csr1*, produces a chlorsulfuron-resistant AHAS activity (7, 10). We have shown that *imr* maps close to the *CSR1* locus and cosegregates with *csr1*. Biochemical studies have suggested that, like the sulfonylurea herbicides, the imidazolinones act by binding to the active site of AHAS (17, 18). Thus, it is very likely that *imr* represents a new allele of *CSR1*. Both the wild type and the chlorsulfuron-resistant alleles of the *CSR1* locus have been cloned and characterized (10, 15). These studies should facilitate the

analysis of the molecular basis of imidazolinone resistance of line GH90. If such molecular analyses prove that *imr* is indeed a *CSR1* allele, it should be useful as a selectable marker in plant molecular genetics or in engineering imidazolinone resistance in various crop species.

The *imr* and *csr1* (10) mutations cause high levels of AHAS resistance to either the imidazolinones or the sulfonylureas, but not to both. Assuming that both classes of herbicides bind the active site of AHAS as suggested (16–18, 22), then *imr* and *csr1* mutations probably cause amino acid substitutions at the active site that prevent the binding of only one herbicide class. That such discrimination could occur is not surprising considering how chemically distinct sulfonylurea compounds are from imidazolinone compounds. An analogous situation has been described previously involving cross-resistance of *C. reinhardtii* mutants to several classes of herbicides which bind the second electron accepting plastoquinone of PSII binding protein (5).

To date, no loss-of-function (auxotrophic) alleles of *CSR1* have been isolated for use in biochemical and physiological studies of branched-chain amino acid biosynthesis. Both *csr1* and *imr* may be useful in the isolation of such auxotrophs (9).

Preliminary results for two additional imazapyr-resistant mutants GH91 and GH92 have suggested that both are a result of a single dominant mutation linked to *GL1* and that both have an imazapyr-resistant AHAS activity. The similarity in phenotype and genotype to GH90, the fact that the three mutants were not independently isolated, and the expected low frequency of imazapyr-resistant mutants in an M2 population (8) suggest that GH91 and GH92 are siblings of GH90.

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