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Sulfonylurea-resistant mutants of *Arabidopsis thaliana*

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Summary. Chlorsulfuron-resistant mutants of *Arabidopsis thaliana* were isolated by screening for growth of seedlings in the presence of the herbicide. Both whole plants and derived tissue cultures were resistant to concentrations of the herbicide approximately 300-fold higher than that required to prevent growth of the wild-type. The resistance is due to a single dominant nuclear mutation at a locus designated *csr* which has been genetically mapped to chromosome-3. Acetohydroxy acid synthase activity in extracts from chlorsulfuron-resistant plants was much less-susceptible to inhibition by chlorsulfuron and a structurally related inhibitor than the activity in wild-type extracts. This suggests that the *csr* locus is the structural gene for acetohydroxy acid synthase.

Key words: Herbicide resistance – Acetolactate synthase – Acetohydroxy acid synthase – Chlorsulfuron – Sulfometuron methyl – Imidazolinone

Introduction

The first enzymatic step common to the biosynthesis of the branched-chain amino acids is catalyzed by acetohydroxy acid synthase (AHAS; E.C.4.1.3.18; also referred to as acetolactate synthase). Although the genetics and biochemistry of this enzyme have been extensively studied in microorganisms (for review see DeFelice et al. 1982) relatively little is known about this and related amino acid biosynthetic enzymes in higher plants (Bryan 1980). Recently, two classes of agriculturally important herbicides, the sulfonylureas and the imidazolinones, have been shown to act by specifically inhibiting AHAS; (LaRossa and Falco 1984; Shaner et al. 1984). A number of the sulfonylurea compounds, which have been the most extensively studied of the two classes, are toxic to both plants and microorganisms. Dominant mutations that confer resistance to the sulfonylurea herbicide sulfometuron methyl in *Salmonella typhimurium* (LaRossa and Schloss 1984) and *Saccharomyces cerevisiae* (Falco and Dumas 1984) have been shown to map to the structural gene for AHAS. In each case the mutations result in the synthesis of a herbicide-resistant AHAS. Analogous mutants have also been isolated in higher plants by selecting for sulfonylurea-resistant mutants in tobacco tissue culture (Chaleff and Ray 1984). Regenerated plants retain the herbicide resistant phenotype due to single dominant mutations which cosegregate with a herbicide-resistant AHAS activity (Chaleff and Mauvais 1984).

Because of the potentially broad utility of dominant herbicide resistance as a selectable marker for genetic studies, we have isolated mutants of *Arabidopsis thaliana* (L.) Heynh. which are highly resistant to sulfonylurea herbicides. The mutants should also be useful in facilitating the genetic and physiological analysis of branched-chain amino acid biosynthesis in higher plants.

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Materials and methods

Lines, growth conditions and genetic methods. All lines of *Arabidopsis thaliana* used in this study except MSU8 were descended from the Columbia wild-type. The mutant line MSU53 (*gl-1*) was isolated following EMS mutagenesis. The mutant line MSU8 (*an, py, gl-1, cer-2, ms, er*), which has at least one genetic marker on each of the five chromosomes, was derived from the Landsberg erecta and was provided by M. Koornneef. Derivation of the mutant line GH50 (*Crs-1*) is described in the text.

The M2 seed used in this study was produced by imbibing approximately 100,000 wild-type seeds in 100 ml of water containing 0.3 ml of ethyl methane sulfonate (Sigma, St. Louis, MO, USA) for 16–18 h at room temperature. At this time the mutagenized (M1) seed was washed several times with water then planted and grown to maturity at a density of approximately 1 plant cm^{-2}. The resulting M2 seed was bulk-harvested. The rationale for the mutagenesis procedure has been described in some detail elsewhere (Haughn and Somerville 1986).

Plants were grown under continuous fluorescent illumination of 90–150 $\mu E\ m^{-2}\ s^{-1}$ at 20–24° C. Methods for the growth of plants in pots, seed harvesting and cross pollination have been described elsewhere (Somerville and Ogren 1982).

Reagents. Chlorsulfuron (2-chloro-*N*-[(4-methoxy-6-methyl-1,3,5-triazin-2-yl) aminocarbonyl] benzenesulfonamide) and sulfometuron methyl (methyl-2-[[[[(4,6-dimethyl-2-pyrimidinyl)amino]-carbonyl]amino]sulfonyl]-benzoate) were generously provided by C.J. Arntzen and R. Giaquinta, E.I. Dupont de Nemours and Co., Wilmington DE, USA. These compounds are also available from Chem Service Inc., West Chester, PA, USA. Imazapyr (AC 252, 925; isopropylamine salt of 2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl) nicotinic acid was generously provided by

D.L. Shaner, American Cynamid Co., Princeton, N.J., USA. This compound is similar to AC243997 described by Shaner et al. (1984). 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); sulfometuron methyl, 0.1 mg ml^{-1} in 10 mM potassium phosphate (pH 7.5); Imazapyr, 50 mM in water.

Media. Plants were grown on a minimal medium consisting of 5 mM KNO_3, 2.5 mM KH_2PO_4 (adjusted to pH 5.5), 2 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 51 μM Fe-EDTA (Sigma EDFS), 70 μM H_3BO_3, 14 μM $MnCl_2$, 0.5 μM $CuSO_4$, 1 μM $ZnSO_4$, 0.2 μM $NaMoO_4$, 10 μM NaCl, 0.01 μM $CoCl_2$ per l.

For most purposes plants were grown in 12.5 cm pots containing a support medium of equal parts of perlite, vermiculite and sphagnum saturated with minimal medium and overlaid with several mm of fine vermiculite. For growth of plants under sterile conditions seeds were surface sterilized for 10 min in 30% (v/v) bleach and 0.02% Triton-X100 and distributed in bacteriological petri plates (90 mm × 23 mm) containing minimal medium supplemented with 5 g/l sucrose and 7 g/l agar (Haughn and Somerville 1986). Sterile herbicides and amino acids were added after autoclaving the media.

Tissue culture media consisted of a Murashige and Skoog (1962) mineral salts with the following additions: 20 g/l sucrose, 0.4 mg/l glycine, 0.1 mg/l nicotinic acid, 1 mg/l thiamine-HCl, 0.1 mg/l pyridoxine-HCl, 100 μM Fe-EDTA and 10 g/l agar. The pH was adjusted to 5.8 with KOH. Plant growth regulators were added to autoclaved medium in the following concentrations: callus induction medium contained 0.23 μM kinetin, 4.52 μM 2,4-dichlorophenoxy acetic acid (2,4-D); and callus maintenance medium contained 0.46 μM kinetin; 2.2 μM 2,4-D.

Tissue culture methods. Seeds were surface sterilized and germinated on solid callus induction medium. After 40 days the resulting callus cultures were fragmented and transferred to callus maintenance medium. Callus was subcultured every 30–40 days. For the measurements of callus growth rate, tissue was subcultured on callus maintenance media overlaid with 7.0 cm Whatman # 1 filters. At periodic intervals the filter papers with callus were removed, weighed and replaced under sterile conditions.

Test for herbicide resistance. The test for herbicide resistance involved surface sterilization of seed and subsequent germination on agar-solidified minimal medium. After 5 to 9 days plants were transferred to the surface of minimal plates containing 100 nM chlorsulfuron. Those plants showing normal growth of both the shoot and root apical meristems after 4 to 7 days were scored as resistant. During genetic mapping experiments the first set of leaves were allowed to emerge prior to transfer to herbicide containing media to permit the *gl-1* marker to be scored on herbicide sensitive plants.

Enzyme assays. The preparation of extracts from *Arabidopsis* leaf tissue and the assay for AHAS activity were done as described by Chaleff and Mauvais (1984) with the following modifications. Four hundred to nine hundred mg of leaf tissue was harvested from 4 week old *Arabidopsis* plants (plants were just beginning to bolt) and homogenized in 2 ml of cold extraction buffer (Polyclar AT was omitted). Following ammonium sulfate fractionation, precipitated protein was dissolved in 0.5 ml of column buffer before desalting by Sephadex G-25 chromatography. The protein-containing fractions were pooled (2–3 ml) and 150 μl of this extract was used in each reaction. After the conversion of acetolactate to acetoin using H_2SO_4 the pH was adjusted to 7.0 with NaOH and precipitate was removed by centrifugation for 5 min in an Eppendorf microfuge. The acetoin concentration in 850 μl of the supernatant was determined by the procedure of (Westerfield 1945) as modified by Chaleff and Mauvais (1984). Protein concentrations were measured using the method of Groves et al. (1968).

Results

Isolation of chlorsulfuron-resistant mutants

The growth of both shoot and root apical meristems of wild-type *Arabidopsis* on agar-solidified mineral media was completely inhibited by chlorsulfuron concentrations of 28 nM or higher, and severely limited by concentrations as low as 10 nM. By contrast, growth was not inhibited by 280 nM chlorsulfuron if 1 mM valine and 1 mM isoleucine were included in the medium. This result is consistent with the results of previous studies in tobacco and pea indicating that growth inhibition by chlorsulfuron was due to inhibition of branched-chain amino acid biosynthesis (Chaleff and Ray 1984; Ray 1984).

Chlorsulfuron-resistant mutants of *Arabidopsis* were isolated by placing approximately 300,000 M2 seeds, at a density of approximately 10,000 seeds per plate, on the surface of agar-solidified mineral medium containing 200 nM chlorsulfuron. After one week of continuous illumination only four of the seeds had germinated and produced green seedlings. These four putative chlorsulfuron-resistant plants (lines GH50–GH53) were transferred to herbicide-free medium in pots and grown to maturity without selection. All of the M3 progeny of lines GH50 and GH52, and 77% of the M3 progeny from line GH51 were resistant to 200 nM chlorsulfuron. M3 progeny of line GH53 were not resistant to 200 nM chlorsulfuron and were not analyzed further.

The mutant line GH50 was chosen for subsequent analysis because it appeared to be homozygous and was the most vigorous of the three chlorsulfuron-resistant lines. In subsequent analyses of the M4 and M5 generations of this line, all of the progeny were found to be resistant to chlorsulfuron, indicating that the trait is stably inherited.

Genetic analysis of GH50. In order to determine the mode of inheritance of the herbicide-resistance, M3 plants from line GH50 were crossed to the genetically marked chlorsulfuron-sensitive lines MSU53 and MSU8. F1 progeny from both crosses were resistant to 100 nM chlorsulfuron (Table 1) and wild-type with respect to the remaining markers. The chlorsulfuron-resistant phenotype segregated 3:1 (resistant:sensitive) among the F2 progeny of both crosses (Table 1). Considered together, these results indicate that resistance is due to a single dominant nuclear mutation at a locus which we have designated *csr-1* (chlorsulfuron resistance). We have designated the allele in GH50 as *Csr-1*[1] (capitalization of the first letter has been used to indicate a dominant allele).

Table 1. Genetic segregation and linkage analysis of chlorsulfuron resistance of line GH50

Line	Number of plants				
	Resistant		Sensitive		
	Observed	Expected[a]	Observed	Expected[a]	X^2
GH50 (selfed)	141	–	0	–	
MSU53 (selfed)	0	–	114	–	
MSU8 (selfed)	0	–	40	–	
GH50 × MSU53 (F1)	28	–	0	–	
MSU8 × GH50 (F1)	31	–	0	–	
GH50 × MSU53 (F2)	374	380	133	127	0.37
MSU8 × GH50 (F2)	610	613.5	208	204.5	0.08
MSU8 × GH50 (F2)					
gl-1/gl-1	51	106	–	–	38.0
+/−	373	318	–	–	
GH50 × MSU53 (F2)					
gl-1/gl-1	37	–	44	–	
+/−	170	–	34	–	

[a] Expected numbers were calculated assuming a 3:1 segregation

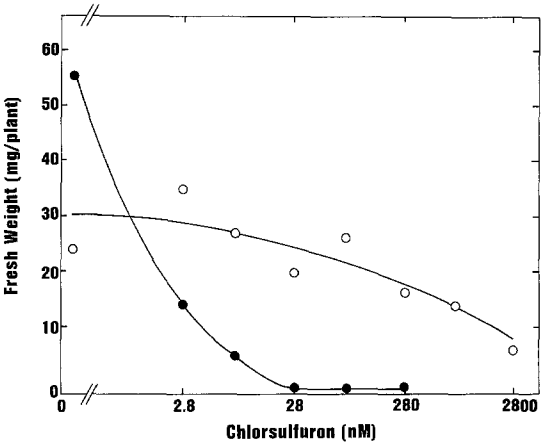

Fig. 1. The growth of wild-type and mutant GH50 on chlorsulfuron. Seeds were surface-sterilized and germinated on minimal medium. After five days seedlings were transferred onto minimal medium (10 plants/plate) containing the indicated concentration of chlorsulfuron. Fresh weight of individual plants was measured 19 days after transfer. Results for wild-type (●—●) represent the average weight of 30 individuals. Results for GH50 (○—○) represent the average weight of 20 individuals. At every chlorsulfuron concentration tested the average fresh weight of wild-type plants was statistically different from that of GH50 at the 0.005 confidence level using the *t*-test

Herbicide-resistant F2 progeny from the MSU8 × GH50 cross were transplanted to soil and scored for each of the six recessive markers of line MSU8 (Table 1). Five of the six markers *an*, *py*, *cer-2*, *er* and *ms-1*, segregated independently of the *crs* locus (3:1 among chlorsulfuron-resistant F2 progeny; data not shown). Only *gl-1*, the marker for chromosome 3, showed linkage to *csr-1* (Table-1; $X^2 = 38.0$, $P < 0.01$). This linkage was confirmed by the *gl-1*, *Csr-1*^{1} digenic ratios in the F2 progeny of cross GH50 × MSU53 (Table 1). The distance was estimated to be 27.3 map units by the method of Suiter et al. (1983).

Whole-plant resistance. In order to measure the level of chlorsulfuron-resistance in whole plants, five day old seedlings of wild-type and GH50 were transferred to agar-solidified medium containing a range of chlorsulfuron concentrations. The mean fresh-weight of the plants after two weeks of growth is shown in Figure 1. Wild type plants were able to grow to maturity (albeit slowly) on 2.8 nM chlorsulfuron but no growth was observed on concentrations of 28 nM or higher. By contrast, plants of line GH50 were able to grow even on the highest concentration of chlorsulfuron tested (2800 nM). Thus, the mutant is resistant to at least 100-fold higher concentrations of the herbicide than that which completely prevents growth of the wild-type. However, it should be noted that in the absence of herbicide, wild type plants were, on basis of a Student's *t*-test, significantly larger than plants from line GH50. This result could be due to either a pleiotropic effect of the mutation causing chlorsulfuron resistance or an effect of an unrelated mutation in the genetic background of line GH50.

Resistance in tissue culture. The expression of chlorsulfuron resistance in tissue culture was examined using callus cultures derived from wild-type, line GH50 and F1 progeny from a GH50 × wild-type cross.

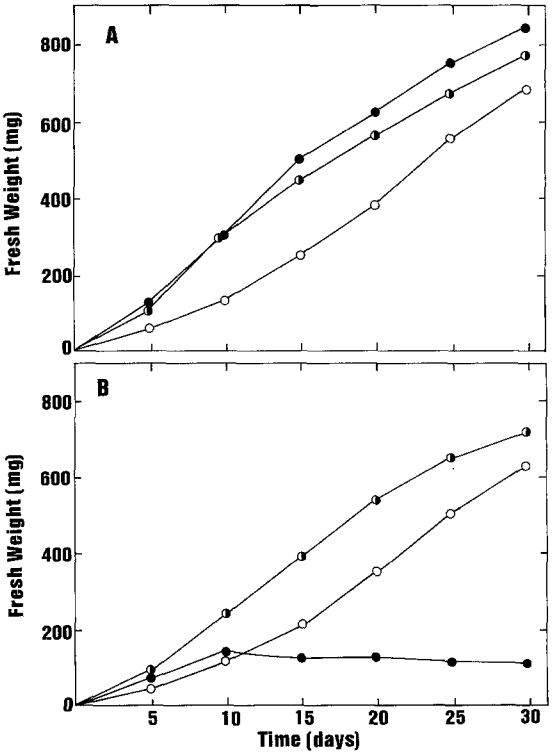

Fig. 2A, B. The effect of chlorsulfuron on the growth of callus derived from herbicide-resistant and sensitive lines of *Arabidopsis*. Aliquots of approximately 80 mg of tissue were subcultured onto plates containing callus maintenance medium and the fresh weight determined at five day intervals. The weight shown is the total increase in fresh weight after transfer and represents the average of four independent replicates. **A** Medium lacked herbicide. **B** Medium contained 300 nM chlorsulfuron. *Symbols*: (●—●) wild-type; (○—○) GH50 (*Csr-1*^{1}); (◑—◑) heterozygous tissue derived from F1 hybrid from a cross between wild-type and GH50

In the absence of herbicide the weight of wild-type tissue increased linearly with time (Fig. 2A). However, in the presence of 300 nM chlorsulfuron wild-type tissue grew only slightly (Fig. 2B) and was necrotic by 20 days. The inhibition of growth was apparently due to inhibition of branched chain amino acid biosynthesis since the addition to the growth medium of 0.5 mM valine and 0.5 mM isoleucine permitted the growth of wild-type tissue even in the presence of 300 nM chlorsulfuron (data not shown). Growth of callus tissue carrying one or two copies of the *Csr-1*^{1} allele was not affected by 300 nM chlorsulfuron (compare Fig. 2A and 2B) demonstrating that the chlorsulfuron resistance of line GH50 is a dominant trait in tissue culture.

There was a noticeable lag in the growth of GH50 callus from 0 to 15 days after which the cells grew at a rate comparable to wild-type (Fig. 2A and B). We believe that this growth deficiency is not a pleiotropic effect of the *Csr-1* mutation for two reasons. First, GH50 cells growing on the same medium supplemented with 0.5 mM isoleucine and 0.5 mM valine showed a similar lag period. Second, cells heterozygous for the *Csr-1*^{1} allele showed no such lag, even in the presence of levels of chlorsulfuron which kill the wild-type. Since these growth conditions render the heterozygote entirely dependent on the *Csr-1*^{1} allele it is apparent that it is not the properties of this allele which are deleterious to growth.

Inhibition of AHAS activity by chlorsulfuron. Chlorsulfuron has been shown to be a specific inhibitor of AHAS in plants (Chaleff and Mauvais 1984). Therefore, the mutation of line GH50 could conceivably cause herbicide resistance by altering the AHAS structural locus such that the enzyme is no longer inhibited by chlorsulfuron. This possibility was tested by measuring the effects of chlorsulfuron on AHAS activity in partially purified extracts of lines GH50, the wild-type and F1 heterozygotes from a wild-type × GH50 cross. The specific activities of AHAS in extracts from all three genotypes were similar (see figure legend to Fig. 3). The addition of 2.8 nM chlorsulfuron inhibited AHAS activity in wild-type extracts by 42%. By contrast 1,000-fold more chlorsulfuron was needed to inhibit 50% of AHAS activity from the herbicide resistant line GH50 (Fig. 3). Such a result could be due to the presence in GH50 extracts of either a herbicide-resistant AHAS activity or an enzyme activity that detoxifies the herbicide. However, approximately 50% of the total AHAS activity in extracts from heterozygous (*Csr-1*^{1}/+) plants was highly resistant to chlorsulfuron (Fig. 3A). This observation is most readily explained by proposing that the *Csr-1*^{1} allele encodes a chlorsulfuron-resistant isoform of AHAS.

Resistance of GH50 to other herbicides. The mutant line GH50 was tested for resistance to two other compounds which inhibit AHAS activity; the sulfonylurea herbicide sulfometuron methyl (SM) and the imidazolinone herbicide Imazapyr. Wild-type seed of *Arabidopsis* was able to germinate and grow on 0.28 nM SM and 50 nM Imazapyr, but not on 2.8 nM SM or 500 nM Imazapyr. Line GH50 was able to germinate and grow (albeit slowly) on 28 nM SM, indicating that the *Csr-1*^{1} allele conferred some resistance to this compound. By contrast line GH50 was not more resistant to Imazapyr as compared to the wild-type. The effects of the *Csr-1*^{1} allele on the in vitro inhibition of AHAS activity by SM and Imazapyr was similar to the effects on growth. AHAS activity in GH50 extracts was

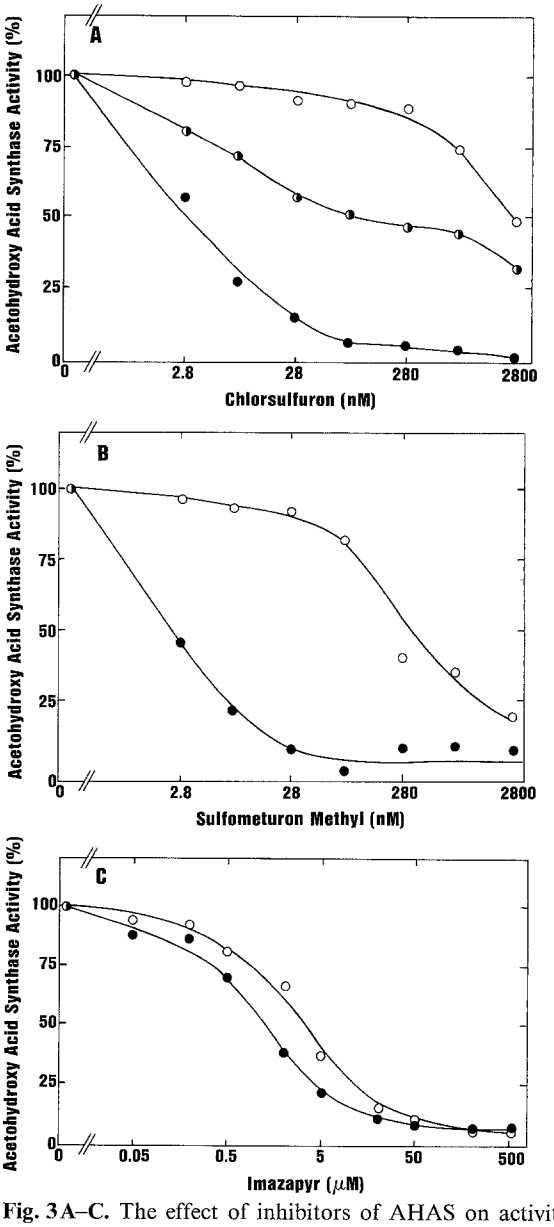

Fig. 3A–C. The effect of inhibitors of AHAS on activities in extracts from chlorsulfuron-resistant and -sensitive lines of *Arabidopsis*. Specific activities (nmol acetoin mg protein^{-1} min^{-1}) were determined in the presence of the indicated concentration of herbicide. Values given are the percent of the specific activity in the absence of herbicide using extracts from line GH50 (o—o), wild-type (●—●) and heterozygous individuals from the wild-type × GH50 cross (◑—◑). **A** The AHAS activities in the absence of chlorsulfuron were wild-type 7.3; GH50, 7.0; and F1 (wild-type × GH50), 6.5. Values presented are the average results obtained in two independent experiments. **B** The AHAS specific activities in the absence of SM were wild-type, 5.2 and GH50, 2.8 **C** The AHAS specific activities in the absence of Imazapyr were wild-type 3.2; GH50, 5.7. Values presented are the average from two independent experiments

100-fold more resistant to SM than activity in wild-type extracts (Fig. 3B) but was not resistant to Imazapyr (Fig. 3C).

Discussion

We have isolated and characterized a line of *Arabidopsis* which is homozygous for a dominant nuclear mutation

(*Csr-1*^{1}) that confers resistance to the sulfonylurea herbicides chlorsulfuron and sulfometuron methyl. The analysis of the effects of sulfonylureas on AHAS activity in extracts from plants carrying the *Csr-1*^{1} allele indicates that resistance is due to a sulfonylurea-resistant enzyme activity. It is likely, therefore, that the *Csr-1*^{1} is an allele of AHAS structural locus and results in an enzyme modification that alleviates inhibition of catalysis by the sulfonylureas. Such an alteration in the enzyme has precedents since mutations conferring resistance to sulfonylureas in microorganisms have been mapped to the structural locus for AHAS (La Rossa and Schloss 1984; Falco and Dumas 1985).

The number of copies of the AHAS gene in the genome of *Arabidopsis* has not been determined. However, as shown here, a single mutation can render all of the AHAS activity in leaf tissue insensitive to inhibition by levels of chlorsulfuron which completely inhibit AHAS activity in extracts from the wild-type. This suggests that there is only one copy of the gene expressed in *Arabidopsis* leaves. Because the growth of non leaf tissues is also resistant to chlorsulfuron, it is apparent that the *csr* locus is also expressed in non-leaf tissues. The evidence that one gene controls AHAS activity in *Arabidopsis* contrasts with the situation in tobacco where mutations at two loci have been shown to confer resistance (Chaleff and Ray 1984). The differences in number of loci may simply reflect the fact that tobacco is a tetraploid.

The chlorsulfuron resistant line GH50 showed significant resistance to another sulfonylurea herbicide, sulfometuron methyl. A similar result was found for sulfonylurea-resistant tobacco lines (Chaleff and Ray 1984). By contrast, line GH50 was not resistant to the structurally dissimilar AHAS inhibitor Imazapyr. Therefore, it may be possible to generate additional alleles of AHAS by selecting mutants resistant to Imazapyr. Imazapyr-resistant mutants may be useful in determining whether the sulfonylurea and imidazolinone compounds inhibit AHAS by different mechanisms.

We have not extensively characterized the other two chlorsulfuron-resistant mutants which were isolated in the same screen that produced line GH50. Preliminary evidence has indicated that chlorsulfuron resistance in these mutants (lines GH51 and GH52) is also due to a dominant nuclear mutation which is linked to the *gl-1* locus, by approximately 25 map units. Therefore, it is likely that all three isolates are allelic. Further, because all three lines were isolated from the same batch of M2 seed, they may have arisen from the same mutational event.

Genes that confer resistance to antibiotics have proven to be invaluable tools as selectable genetic markers in bacteria. By analogy, genes that confer herbicide resistance could be important as markers for plant molecular genetics. Because the *Csr-1*^{1} allele is dominant and nuclear, the corresponding gene should be useful in this respect. Since resistant mutants occur at a low frequency, even in a highly mutagenized population, spontaneous mutants should not be a significant problem in any selection protocols employing the *csr* gene as a selectable marker. In addition, the mutation results in a high level of chlorsulfuron resistance expressed at both the tissue culture and whole plant levels. Assuming that *Csr-1*^{1} defines an allele of the gene encoding AHAS, it should be possible to clone the altered gene for use as a genetic marker in plant transformation experiments.

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