Mol Gen Genet (1988) 211:266–271

MGG

Transformation with a mutant *Arabidopsis* acetolactate synthase gene renders tobacco resistant to sulfonylurea herbicides

George W. Haughn[1], Julie Smith[2], Barbara Mazur[2], and Chris Somerville[1]

[1] MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI 48824, USA
[2] Central Research and Development Department, Du Pont Experimental Station 402, Wilmington, DE 19898, USA

Summary. A gene encoding acetolactate synthase was cloned from a chlorsulfuron-resistant mutant of *Arabidopsis*. The DNA sequence of the mutant gene differed from that of the wild type by a single base pair substitution. When introduced into tobacco by Ti plasmid-mediated transformation the gene conferred a high level of herbicide resistance. These results suggest that the cloned gene may confer agronomically useful levels of herbicide resistance in other crop species, and that it may be useful as a selectable marker for plant transformation experiments.

Key words: Ti plasmid – Chlorsulfuron – Transformation – Acetohydroxy acid synthase

Introduction

Herbicides which show low toxicity to crop species play an important role in the control of weeds in modern agriculture. However, such selective herbicides are often available only for the major crop species because of the high costs of development. An alternative to the identification of new selective herbicides is the genetic modification of susceptible crop species so that they are resistant to relatively nonselective herbicides. One method of achieving this is through the genetic transformation of plants to herbicide resistance. The prerequisites for such an approach are the ability to transform the species of interest, and the availability of a gene which confers herbicide resistance.

One potential source of genes encoding herbicide resistance is the small crucifer *Arabidopsis thaliana* L.. This species has a number of useful characteristics (Estelle and Somerville 1986; Meyerowitz and Pruitt 1985) which facilitate the direct selection of rare herbicide-resistant mutants at the whole plant level. Using such an approach we have been able to identify a mutant line of *Arabidopsis* (Haughn and Somerville 1986, 1987) which is highly resistant to at least two different sulfonylurea herbicides (chlorsulfuron and sulfometuron methyl). The sulfonylureas are a group of compounds which inhibit the branched-chain amino acid biosynthetic enzyme acetolactate synthase (ALS) (LaRossa and Schloss 1984; LaRossa and Falco 1984). Herbicide resistance in the *Arabidopsis* mutant segregated as a single, dominant, nuclear mutation and co-segregated with sulfonylurea-resistant ALS activity (Haughn and Somerville 1986). Mutations causing similar phenotypes in yeast and bacteria have been shown to result from single base pair substitutions within an ALS gene (Yadav et al. 1986). Therefore, we reasoned that the dominant nuclear mutation in the *Arabidopsis* line might also be within an ALS gene.

An ALS gene had been isolated from a genomic library of wild-type *Arabidopsis* DNA through its nucleic acid homology to the ALS gene from the yeast *Saccharomyces cerevisiae* (Mazur et al. 1987). DNA sequence analysis showed that the *Arabidopsis* gene contained an uninterrupted 2 kb open reading frame. The deduced amino acid sequence of the gene had several regions with high homology to the deduced sequences of ALS from yeast and from *Escherichia coli* (Mazur et al. 1987). Southern blot analyses suggested that the cloned gene represented the only ALS gene in *Arabidopsis*, a finding consistent with genetic studies of the *Arabidopsis* chlorsulfuron-resistant mutant. Here we report the isolation of the mutant ALS gene from the herbicide-resistant line and the use of the gene to transform tobacco to a chlorsulfuron-resistant phenotype.

Materials and methods

Bacterial strains, phage, plasmids and plant lines. E. coli strains used were: HB101 (F^- *hsdR hsdM recA13 supE44 lacZ4 leuB6 proA2 thi strA*), GM161 (F^- *thr-1 leuB6 dam-4 thi-1 hsdS1 lacY tonA21 supE44*), and JM83 (F^- *ara thi strA Δ[lac-proAB] ϕ80dlacZ ΔM15*). *Agrobacterium tumefaciens* strain C58C1rif^r (pGV3850) (Zambryski et al. 1983) was used in all plant transformation experiments. The intermediate vector pMON200 was obtained from the Monsanto Co. (Rogers et al. 1986). The phage vector λ2001 was obtained from S. Brenner (Karn et al. 1984).

The chlorsulfuron-resistant *Arabidopsis* line GH50 was derived from the Columbia wild type (Haughn and Somerville 1986). In keeping with the recommendations of the ad hoc committee on genetic nomenclature in *Arabidopsis* we have renamed the mutant locus in this line *csr1*, and the specific allele *csr1-1*. The *Nicotiana tabacum* line designated MSU223 is a streptomycin-resistant line of cv. Petit Havana, originally designated str-r1 (Maliga et al. 1973).

Growth of plants and callus cultures. Growth conditions for *Arabidopsis* plants have been previously described (Haughn and Somerville 1986). Similar conditions were used to grow tobacco plants. Tissue culture media consisted of MS salts (Gibco) with the following additions: 30 g/l sucrose,

Offprint requests to: C. Somerville

0.4 mg/l glycine, 0.1 mg/l nicotinic acid, 1 mg/l thiamine, 0.1 mg/l pyridoxine, 10 mg/l inositol, 7 g/l agarose. The pH was adjusted to 5.7 with KOH. Plant growth regulators, chlorsulfuron (Chem Services Inc., PO Box 3108, West Chester, PA 19381) and antibiotics were added to autoclaved media. Callus induction medium contained 0.23 μM kinetin and 4.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D). Callus maintenance medium contained 0.46 μM kinetin and 2.2 μM 2,4-D. Shoot induction medium contained 1 mg/l benzyladenine and 0.1 mg/l α-naphaleneacetic acid (NAA). Root induction medium contained one-tenth the concentration of MS major salts, normal concentration of MS minor salts (Haughn and Somerville 1986), 5 g/l sucrose and no hormones. Shoots were induced to form roots by dipping the basal end into 0.1 mg/ml NAA before transferring to root induction medium.

Nucleic acid techniques. Standard techniques were used for growth of phage and bacteria and for routine manipulations of DNA (Maniatis et al. 1982). For experiments involving methylation-sensitive restriction enzymes, plasmids were propagated in *E. coli* strain GM161. *Arabidopsis* DNA was isolated by the method of Leutwiler et al. (1984). *Agrobacterium* DNA was isolated by the method of Dhaese et al. (1979). Tobacco DNA was prepared as described by Buchholz and Thomashow (1984) except that sarkosyl and sodium lauryl sulfate were omitted from the lysis buffer.

For Southern analysis, DNA was cleaved with restriction endonucleases, resolved by electrophoresis in 0.8% agarose gels and partially hydrolyzed by soaking the gel in 0.25 M HCl for 30 min at room temperature. The gel was soaked in 0.4 M NaOH for 20 min, then the DNA was transferred to a nylon membrane (Zeta-Probe; BioRad) for 12 h using 0.4 M NaOH as the transfer solution. The filters were prehybridized 10 to 12 h at 68° C in 6 × SSPE (SSPE is 0.15 M NaCl, 10 mM NaH_2 PO_4, 1 mM EDTA adjusted to pH 7.4 with NaOH), 5 × Denhardt's solution (Maniatis et al. 1982), 0.5% SDS (w/v) and 200 μg/ml denatured herring DNA (Sigma). The filters were hybridized for 24 h at 68° C in the same solution which also contained 10% (w/v) dextran sulfate and 10^7 dpm/ml of ^{32}P-labeled probe which was nick translated to a specific activity of approximately 10^8 dpm/μg DNA. The filters were washed twice for 15 min in 2 × SSC (SSC is 0.15 M NaCl, 15 mM sodium citrate, pH 7.0), 0.5% SDS at 23° C, once for 15 min in 2 × SSC, 0.1% SDS at 23° C then twice (for 2 h and 30 min, respectively) at 68° C in 0.1 × SSC, 0.5% SDS.

Construction and screening of an Arabidopsis genomic library. Total DNA from *Arabidopsis* line GH50 was partially digested with *Eco*RI and fragments of 9 to 23 kb were isolated following electrophoresis in 0.8% (w/v) low melting agarose gels. Contaminants from the agarose were removed by chromatography of the DNA on small NACS columns (Bethesda Research Labs, Gaithersburg Md). The *Arabidopsis* DNA was ligated in the presence of *Eco*RI-digested λ2001 DNA and packaged with commercially available extracts (Promega). Recombinant bacteriophage were selected by growth and amplification on *E. coli* strain NM539 (Frischauf et al. 1983). Bacteriophage with inserts homologous to the *Arabidopsis* ALS gene were identified by plaque hybridization (Maniatis et al. 1982). The nitrocellulose filters were prehybridized for 11 h at 42° C in a solution containing 5 × SSPE, 5 × Denhardt's solution, 0.1% SDS, 100 μg/ml denatured herring DNA and 50% (v/v) formamide. The DNA fragment to be used as a probe was purified from a low melting agarose gel, labeled with ^{32}P to a specific activity of approximately 10^8 dpm/μg by nick translation then added to the prehybridization solution at 10^7 dpm/ml. Hybridization was performed at 41° C for 21 h. The filters were washed twice for 15 min in 2 × SSC, 0.1% SDS at 23° C, and twice for 1 h in 1 × SSC, 0.1% SDS at 68° C.

DNA sequence analysis. The 5.8 kb *Xba*I fragment from λcsr-1 was subcloned into plasmid pUC19 (Yanisch-Perron et al. 1985) to create plasmid pGH1. The 2.1 kb *Eco*RI fragment and 2.35 kb *Eco*RI-*Xba*I fragment from pGH1 were subcloned into M13 vectors M13mp18 and M13mp19 (Yanisch-Perron et al. 1985) and the DNA sequence determined by the chain termination method (Sanger et al. 1977).

Plant transformation. A. tumefaciens strains with plasmids pGH6 or pMON200 integrated into the T-DNA of the Ti plasmid pGV3850 were constructed by a triparental mating procedure (Rogers et al. 1986). Parental strains were HB101(pRK2013, Kan^r), JM83(pGH6 or pMON200, Spc^r) and C58C1rif^r(pGV3850). The desired recombinants were selected as colonies resistant to 50 μg/ml rifampicin and 100 μg/ml spectinomycin, and were verified by Southern analysis.

Tobacco was transformed by the leaf disc method (Rogers et al. 1986) using surface-sterilized leaves of *N. tabacum* line MSU223 without nurse cultures. Transformants were selected on media containing 200 μg/ml cefotaxime (Calbiochem) and 300 μg/ml kanamycin. The presence of opines in transformed tissues was assayed as described by Rogers et al. (1986).

Enzyme assays. The preparation of extracts from tobacco callus or leaf tissue and the assays for ALS activity were performed as described (Chaleff and Mauvais 1984), with the following modifications. Three grams of tissue were homogenized in 8 ml of cold extraction buffer (pH 6.5) containing 700 mg of polyvinylpolypyrrolidone (PVPP). The lower pH improves the binding of polyphenols to PVPP. Following ammonium sulfate fractionation, precipitated protein was dissolved in 0.5 ml of buffer and desalted by Sephadex G-25 chromatography. The protein-containing fractions were pooled (2-3 ml) and 150 μl of this extract was used in each enzyme assay. After the conversion of acetolactate to acetoin using H_2SO_4, the pH of each reaction was carefully adjusted to pH 7.0 using 25% NaOH. Following centrifugation at 13000 *g* for 10 min the acetoin concentration in 850 μl of the supernatant was determined as described. The 5% (w/v) α-naphthol (Sigma) solution was prepared within minutes of use since it discolors rapidly.

Results

Isolation and DNA sequence analysis of the ALS gene

A genomic library of total DNA from the herbicide-resistant *Arabidopsis* line GH50 was constructed using the lambda vector λ2001. The library was screened with a fragment of the ALS gene cloned from wild-type *Arabidopsis* (Mazur et al. 1987). Of the 50000 phage screened 7 hybridized to a 2.1 kb *Eco*RI restriction fragment that contained

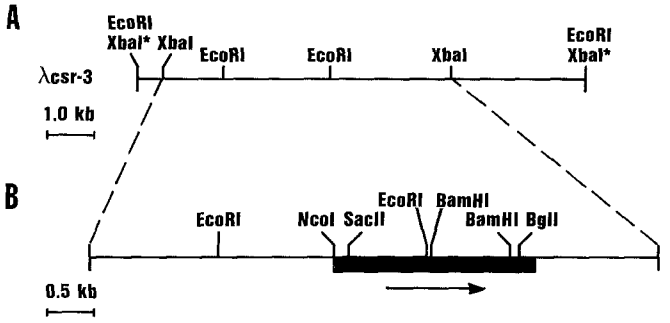

Fig. 1 A, B. Restriction map of the acetolactate synthase (ALS) locus from *Arabidopsis* line GH50. **A** A partial restriction map of *Arabidopsis* DNA carried by one of the recombinant bacteriophage (λcsr-3) identified from a genomic library of *Arabidopsis* line GH50. The *asterisks* indicate restriction sites generated as a result of cloning into the vector λ2001. *B* A more detailed restriction map of the 5.8 kb *Xba*I fragment form λcsr-3. The fragment was subcloned into the *Xba*I site of plasmid pUC19 to create plasmid pGH1. The position of the ALS open reading frame (*solid rectangle*) and the direction of transcription (*arrow*) were deduced by comparing the restriction map with that established for the wild-type gene

the 5' end of the wild-type ALS gene (see Fig. 1). Restriction endonuclease and filter hybridization analyses of the DNA from the seven phage indicated that each carried one of three different but overlapping segments of *Arabidopsis* DNA, and that these segments could be aligned with the physical map of the *Arabidopsis* ALS locus. One bacteriophage (λcsr-3) was chosen for further analysis (Fig. 1).

The designated ALS gene was subcloned away from its flanking DNA into plasmid and M13 vectors. This gene, including 500 bp of 5' and 300 bp of 3' flanking regions was then sequenced on both strands. When the sequence of the mutant gene was compared with that of the wild-type ALS gene from *Arabidopsis*, a single base pair change was found. This change, a C to T transition at nucleotide 870 (Mazur et al. 1987), predicts a proline to serine substitution in the amino acid sequence of ALS as shown in Fig. 2. This change occurs at the same position and is identical to a mutation which confers sulfonylurea resistance in a yeast ALS gene (Yadav et al. 1986). A comparison of the deduced amino acid sequences which flank the altered prolines in the two enzymes is also shown in Fig. 2.

Construction of transgenic tobacco lines carrying the ALS gene

The ALS gene from GH50 was introduced into a herbicide-sensitive line of tobacco to test its ability to transform plants to a herbicide-resistant phenotype. Tobacco was chosen because of the ease with which genes can be introduced by Ti plasmid-mediated transformation. A 5.8 kb *Xba*I fragment containing the ALS coding region with 2.7 kb upstream and 1.3 kb of downstream flanking sequence (Fig. 1 B) was cloned into a Ti plasmid intermediate vector, pMON200, to produce plasmid pGH6. An *A. tumefaciens* strain containing the intermediate vector integrated into the T-DNA of the disarmed Ti plasmid pGV3850 was selected following a triparental mating. Transformation of tobacco leaf discs with recombinant *A. tumefaciens* gave rise to callus resistant to 300 μg/ml kanamycin. Shoots that were subsequently regenerated from the kanamycin-resistant callus were then rooted, transferred to soil and grown to maturity. Three out of the ten regenerated plants were identified as transformants by the presence of nopaline in leaf extracts and by the ability of callus initiated from leaf tissue to grow in the presence of 300 μm/ml kanamycin (Rogers et al. 1986).

Filter hybridization analyses using pGH6 (which carries the right T-DNA border) as a hybridization probe confirmed that T-DNA had integrated in the genome of the putative transgenic plants (Fig. 3). In each of the transformants, the loss of both the 3.2 kb *Hin*dIII right border fragment and the 12.6 kb *Hin*dIII internal fragment suggested that the transferred DNA terminated at the right T-DNA border within the plasmid pGH6. Two of the transformants (GH101 and GH102; Fig. 3, lanes 3 and 4) have a *Hin*dIII border fragment of similar size and may have derived from a single transformation event. The third transformant (GH104, Fig. 3, lane 5) has a more complex *Hin*dIII restriction pattern which suggests that several cop-

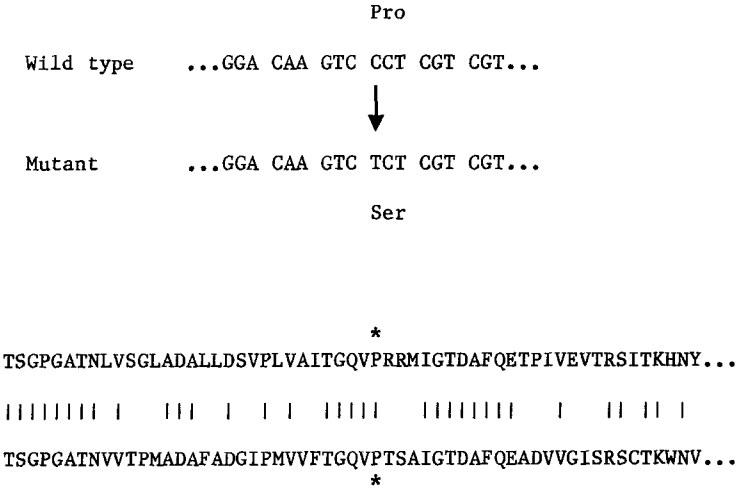

Fig. 2. Identification of the mutation which confers herbicide resistance. A portion of the DNA sequence of the *Arabidopsis* acetolactate synthase (ALS) gene showing the single nucleotide differences in the mutant gene and the resulting deduced amino acid change in the enzyme is presented at the top of the figure. Below this is shown a comparison of the deduced amino acid sequences flanking the altered residues in the *Arabidopsis* and yeast ALS proteins. The *asterisks* designate the proline residues which are replaced in the herbicide-resistant enzymes

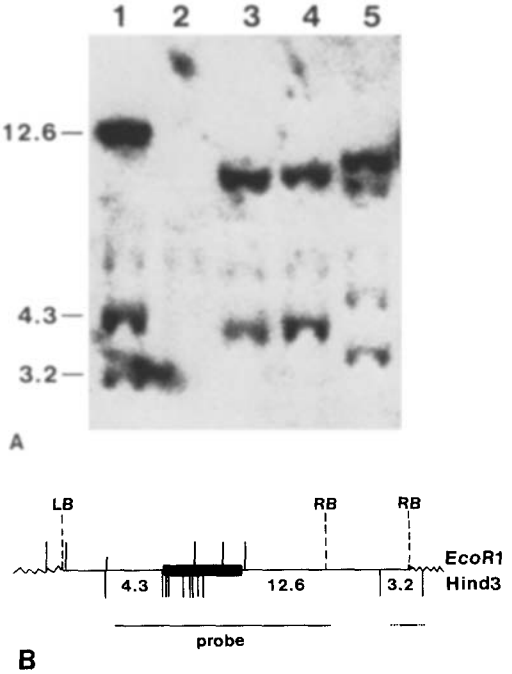

Fig. 3A, B. Filter hybridization analysis of transgenic tobacco plants. **A** Total tobacco or *Agrobacterium tumefaciens* DNA was digested with *Hin*dIII, size fractionated on an agarose gel, transferred to a nylon membrane, hybridized to ^{32}P-labeled plasmid pGH6 [pMON200 containing the 5.8 kb *Xba*I fragment which spans the *Arabidopsis* acetolactate synthase (ALS) gene] and exposed to film. Lane 1, 10 µg of untransformed tobacco DNA plus 80 ng of DNA from *A. tumefaciens* carrying the Ti plasmid construct pGV3850::pGH6; lane 2, 10 µg of untransformed tobacco DNA; lanes 3,4 and 5, 10 µg of DNA from transgenic tobacco lines transformed with the *Arabidopsis* ALS gene (from isolates GH101, GH102, GH104, respectively). Numbers along the ordinate represent sizes of DNA fragments in kilobases. **B** Restriction map of the pGV3850::pGH6 T-DNA region. *Solid vertical lines* indicate positions of *Eco*RI or *Hin*dIII restriction endonuclease sites. *Dashed vertical lines* indicate the positions of the left (LB) and the two right (RB) T-DNA border sequences. The *horizontal bar* represents *Arabidopsis* DNA. The regions of homology to the fragment of plasmid pGH6 DNA used as a hybridization probe are indicated. The sizes in kilobases and positions of the three *Hin*dIII fragments detected in **A**, lane 1, are given. The 3.2 kb *Hin*dIII fragment has homology to the right border sequences present on plasmid pGH6

ies of T-DNA are present or that DNA rearrangements may have occurred during the transformation process. A faint band in the DNA from untransformed tobacco (Fig. 3, lane 2) may be due to hybridization between the *Arabidopsis* and tobacco ALS genes (Mazur et al. 1987).

In order to estimate the number of copies of the *Arabidopsis* ALS gene in the transgenic tobacco plants, a reconstruction experiment was performed. Using an average value of 3.3×10^9 bp as the size of the haploid tobacco genome (Merlo et al. 1980; Zimmerman and Goldberg 1977), then 5.4 pg of pGH6 DNA contains the same number of copies of the 2.1 kb *Eco*RI fragment from the ALS gene as would be found in 1 µg of DNA from a tobacco plant with one copy of the fragment per haploid genome. Southern blots containing DNA from the three transgenic tobacco lines and amounts of pGH6 DNA equivalent to 2 or 10 copies of the ALS genome per haploid genome were probed with the 2.1 kb *Eco*RI fragment of

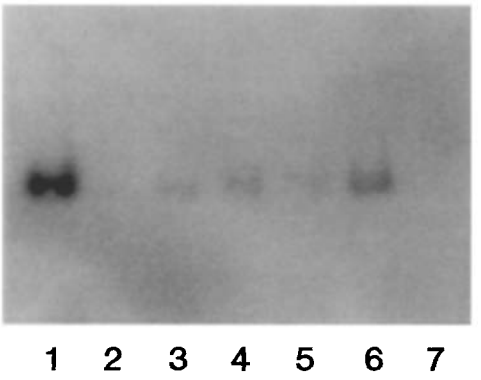

Fig. 4. Analysis of the number of copies of the *Arabidopsis* acetolactate synthase (ALS) gene in the transgenic tobacco plants. Total DNA from tobacco was cleaved with *Eco*RI, electrophoresed in an agarose gel and transferred to a nylon membrane. The filter was probed with the 2.1 kb *Eco*RI fragment containing the 5' region of the ALS gene. Lane 1, 0.38 ng of pGH6 DNA (equivalent to 10 copies per haploid genome) +7 µg MSU223 DNA; lane 2, 7 µg of MSU223 DNA; lane 3, 7 µg GH102 DNA; lane 4, 7 µg GH101 DNA; lane 5, 5 µg GH104 DNA; lane 6, 75 pg pGH6 DNA +7 µg MSU223 DNA; lane 7, 0.38 ng pMON200 DNA + 7 µg MSU223 DNA

the ALS gene (Fig. 4). Note that, less DNA from line GH104 was loaded on the gel than for the other lines. The results of this experiment indicated that the three heterozygous transformed lines GH101, GH102 and GH104 each had less than two copies of the ALS gene per haploid genome.

Herbicide resistance in transgenic plants

The transgenic tobacco plants carrying the *Arabidopsis* ALS gene were shown to have a chlorsulfuron-resistant phenotype by transferring callus derived from leaf tissue to callus maintenance media containing chlorsulfuron at concentrations of 1, 10 and 100 nM. Untransformed tobacco callus and kanamycin-resistant callus from a control transgenic tobacco line GH103 (carrying the intermediate vector pMON200 without the ALS gene) grew on 1 nM chlorsulfuron but not on 10 nM chlorsulfuron. In contrast, transgenic tissue from each of the plants containing the *Arabidopsis* ALS gene could grow in the presence of chlorsulfuron concentrations of at least 100 nM.

Chlorsulfuron resistance at the whole plant level was assayed by growing progeny of the transformed plants on minimal media containing a range of concentrations of the herbicide. The untransformed and vector-transformed control lines were able to grow slowly on 10 nM but not on 100 nM chlorsulfuron. The majority of progeny from transformants carrying the ALS gene, however, were over 100-fold more resistant to chlorsulfuron. These plants grew well at chlorsulfuron concentrations up to 1000 nM, and slowly on concentrations as high as 10000 nM chlorsulfuron (Fig. 5).

Genetic analysis

The mode of inheritance of the chlorsulfuron resistance trait was established by scoring the progeny of self-fertilized transgenic plants. Lines carrying the *Arabidopsis* ALS gene gave rise to both resistant and sensitive progeny in a ratio of approximately 3 to 1 (resistant:sensitive; GH101 179:58;

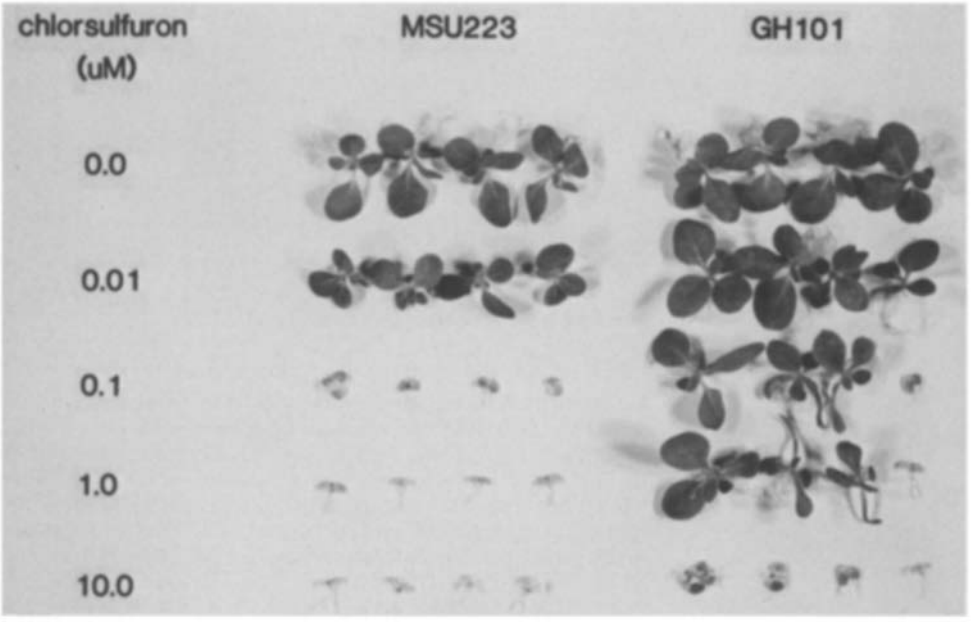

Fig. 5. Chlorsulfuron resistance at the whole plant level in progeny of untransformed (MSU223) and transgenic (GH101) tobacco plants. Seeds were germinated under sterile conditions on minimal agar medium. Six days after planting, seedlings were transferred to minimal medium containing the indicated concentrations of chlorsulfuron. The figure shows representative samples of plants from each treatment photographed 2 weeks after transfer

GH102 179:58; GH104 175:50). This suggests that all of the expressed copies of the ALS gene are inserted at a single nuclear locus and is consistent with the data from Southern blot analyses of the transgenic plants (Fig. 3). Growth of the resistant progeny of individual plants on 100 to 1000 nM chlorsulfuron was not uniform. More than 50% of the plants grew slowly and produced narrow leaves in the presence of 100 or 1000 nM chlorsulfuron (Fig. 5). This variability in response to the herbicide could be due to differences in gene dosage in plants which are either homozygous or heterozygous for the *Arabidopsis* gene, segregation of background mutations introduced during transformation, or physiological variation. These possibilities may be resolved by further genetic analyses once a line homozygous for the introduced ALS gene has been established.

Inhibition of ALS activity by chlorsulfuron

The evidence presented above is consistent with the hypothesis that the mutant ALS gene confers herbicide resistance by encoding a chlorsulfuron-resistant ALS enzyme. To confirm this hypothesis, the effect of chlorsulfuron on ALS activity was measured in extracts of callus tissues derived from the three transformants (Fig. 6). As has been previously reported for suspension cultures (Chaleff and Mauvais 1984), ALS activity in crude extracts of callus tissue established from leaves of wild-type tobacco plants was inhibited more than 85% by chlorsulfuron concentrations above 300 nM. In contrast, this concentration of chlorsulfuron inhibited less than 65% of total ALS activity in extracts of callus derived from leaves of transgenic plants. Equivalent results were obtained using extracts derived from leaf tissue (results not presented). Thus, the *Arabidopsis* ALS gene encodes a fraction comprising about 20% of total ALS activity that is highly resistant to chlorsulfuron. The remaining herbicide-sensitive ALS activity is thought to be the product of the two pairs of *N. tabacum* ALS genes (Chaleff and Ray 1984; Chaleff and Mauvais 1984; Mazur et al 1987). In the absence of herbicide, the amount of ALS activity in extracts of tissue from transgenic plants was consistently lower than that in extracts from wild-type tissue (Fig. 6). This suggests that overexpression of ALS in the transgenic lines does not contribute to chlorsulfuron resistance. The reason for the slightly reduced ALS activity in the transgenic lines is not known.

Discussion

We have cloned an ALS gene from a chlorsulfuron-resistant line of *Arabidopsis*. Two lines of evidence indicate that this gene represents the chlorsulfuron resistance locus *csr1* (Haughn and Somerville 1986). First, the nucleotide sequence of the gene differs from the wild-type gene by a single base pair substitution. This mutation causes a change in the amino acid sequence of ALS which is identical to a mutation in the yeast ALS gene which causes a sulfonylurea-resistant phenotype (Yadav et al. 1986). Second, when transformed into tobacco, the gene confers a herbicide-resistant phenotype and specifies a chlorsulfuron-resistant ALS activity as measured in extracts of callus or whole plants. The high degree of herbicide resistance of transgenic plants carrying the *Arabidopsis* ALS gene suggests that it will prove to be a useful selectable marker for plant molecular genetic studies.

As we did not restructure the *Arabidopsis* ALS gene before it was introduced into tobacco, it seems likely that the gene is expressed in tobacco under the control of its native transcription and translation signals. Thus, it may be possible, by direct selection in tobacco, to clone other *Arabidopsis* genes that confer a dominant resistance phenotype. The small size of the *Arabidopsis* genome (Leutwiler et al. 1984), the high efficiency of transformation and regeneration in tobacco (Rogers et al. 1986) and the use of cosmid Ti binary vectors (An et al. 1985) should make such "shotgun cloning" feasible.

Previous studies have demonstrated that herbicide tolerance can be achieved in transgenic plants by gene overexpression (Shah et al. 1986) or by introduction of mutant bacterial genes (Comai et al. 1985). We have now shown that herbicide-resistant transgenic plants can also be produced using a mutant plant gene. The fact that the *Arabidopsis* gene confers a high degree of resistance in a distantly

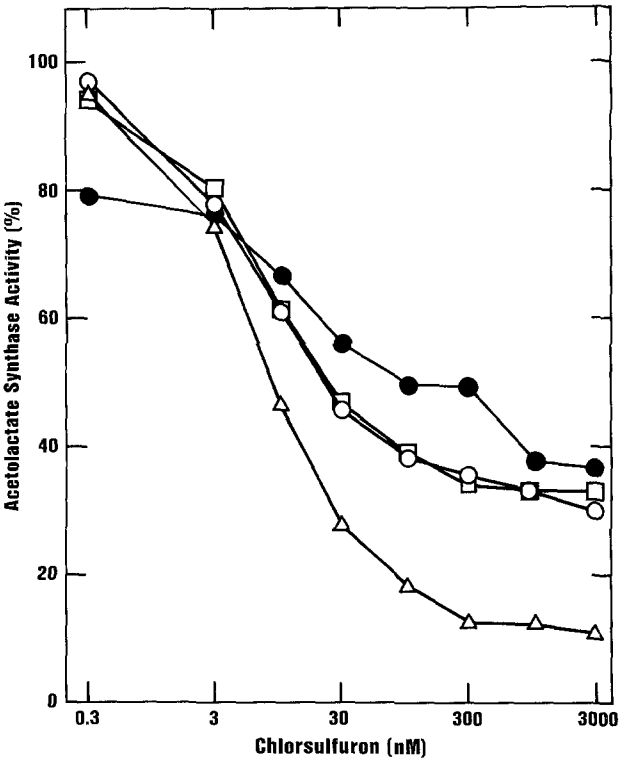

Fig. 6. Effects of chlorsulfuron on acetolactate synthase (ALS) activities in extracts from tobacco. Callus cultures were initiated from leaf tissue on callus induction medium, amplified on callus maintenance medium and assayed for ALS activity. Each point represents the average of results obtained with at least three independently prepared extracts. The specific ALS activities and standard deviations determined in the absence of chlorsulfuron for untransformed tobacco (MSU223), and for transgenic plants GH101, GH102 and GH104 were 107 ± 18, 72 ± 24, 70 ± 24 and 53 ± 13 nmol of acetoin per milligram protein per hour, respectively. ALS activities are presented as percentage activity in the absence of herbicide. Symbols: (△) MSU223, (□) GH101, (○) GH102, (●) GH104

related genus raises the possibility that the gene may be used for genetically engineering sulfonylurea resistance in a wide variety of crop species.

Acknowledgements. We thank S. Rogers for providing pMON200, S. Brenner for the vector λ2001, and J. Schell for *A. tumefaciens* C58C1rif^r(pGV3850). We thank J. Browse, T. Caspar, R. Finkelstein, L. Kunst, J. Martinez-Zapater and B. Moffat for critical comments on the manuscript. This was supported in part by grants from the National Science Foundation (#PCM 8351595) and the U.S. Department of Energy (#DE-ACO2-76ERO1338).

References

An G, Watson BD, Stachel S, Gordon MP, Nester EW (1985) New cloning vectors for transformation of higher plants. EMBO J 4:277–281

Buchholz WG, Thomashow MF (1984) Comparison of T-DNA oncogene complements of *Agrobacterium tumefaciens* tumor-inducing plasmids with limited and wide host ranges. J Bacteriol 160:319–326

Chaleff RS, Mauvais CJ (1984) Acetolactate synthase is the site of action of two sulfonylurea herbicides in higher plants. Science 224:1443–1445

Chaleff RS, Ray TB (1984) Herbicide-resistant mutants from tobacco cell cultures. Science 223:1148–1151

Comai L, Facciotti D, Hiatt WR, Thompson G, Rose RE, Stalker DM (1985) Expression in plants of a mutant *aroA* gene from *Salmonella typhimurium* confers tolerance to glyphosate. Nature 317:741–744

Dhaese P, Degreve H, Decraemer H, Schell J, Van Montagu M (1979) Rapid mapping of transposon insertion and deletion mutations in the large Ti-plasmids of *Agrobacterium tumefaciens*. Nucleic Acids Res 7:1837–1849

Estelle MA, Somerville CR (1986) The mutants of *Arabidopsis*. Trends Genet 2:89–93

Frischauf AM, Lehrach H, Poustka A, Murray N (1983) Lambda replacement vectors carrying polylinker sequences. J Mol Biol 170:827–842

Haughn GW, Somerville CR (1986) Sulfonylurea-resistant mutants of *Arabidopsis thaliana*. Mol Gen Genet 204:430–434

Haughn GW, Somerville CR (1987) Selection for herbicide resistance at the whole-plant level. In: Lebaron HM, Mumma RO, Honeycutt RC, Duesing JH (eds) Biotechnology in Agricultural Chemistry. American Chemical Society, Washington, DC, pp 98–107

Karn J, Matthes HWD, Gait MJ, Brenner S (1984) A new selective phage cloning vector λ2001, with sites for XbaI, BamHI, HindIII, EcoRI, SstI and XhoI. Gene 32:217–224

LaRossa RA, Falco SC (1984) Amino acid biosynthetic enzymes as targets of herbicide action. Trends Biotechnol 2:158–161

LaRossa RA, Schloss JW (1984) The sulfonylurea herbicide sulfometuron methyl is an extremely potent and selective inhibitor of acetolactate synthase in *Salmonella typhimurium*. J Biol Chem 259:8753–8757

Leutwiler LS, Hough-Evans BR, Meyerowitz EM (1984) The DNA of *Arabidopsis*. Mol Gen Genet 194:15–23

Maliga P, Breznovits AS, Marton L (1973) Streptomycin-resistant plants from callus cultures of haploid tobacco. Nature New Biol 244:29–30

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Mazur BJ, Chui CF, Smith JK (1987) Isolation and characterization of plant genes coding for acetolactate synthase, the target enzyme for two classes of herbicides. Plant Physiol 85: 1110–1117

Merlo DJ, Nutter RC, Montoya AL, Garfinkl DJ, Drummond MH, Chilton MD, Gordon MP, Nester EW (1980) The boundaries and copy numbers of Ti plasmid T-DNA vary in crown gall tumors. Mol Gen Genet 177:637–643

Meyerowitz EM, Pruitt RE (1985) *Arabidopsis thaliana* and plant molecular genetics. Science 226:1214–1218

Rogers SG, Horsch RB, Fraley RT (1986) Gene transfer in plants: production of transformed plants using Ti plasmid vectors. Methods Enzymol 118:627–640

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467

Shah DM, Horsch RB, Klee HJ, Kishore GM, Winter JA, Tumer NE, Hironaka CM, Sanders PR, Gasser CS, Aykent S, Siegel NR, Rogers SG, Fraley RT (1986) Engineering herbicide tolerance in transgenic plants Science 233:478–481

Yadav N, McDevitt RE, Benard S, Falco SC (1986) Single amino acid substitutions in the enzyme acetolactate synthase confer resistance to the herbicide sulfometuron methyl. Proc Natl Acad Sci USA 83:4418–4422

Yanisch-Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103–119

Zambryski P, Joos H, Genetello C, Leemans J, Van Montagu M, Shell J (1983) Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. EMBO J 2:2143–2150

Zimmerman JL, Goldberg RB (1977) DNA sequence organization in the genome of *Nicotiana tabacum*. Chromosoma 59:227–252

Communicated by G.R. Fink

Received September 6, 1987