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Comparison of Increased Expression of Wild-Type and Herbicide-Resistant Acetolactate Synthase Genes in Transgenic Plants, and Indication of Posttranscriptional Limitation on Enzyme Activity

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ABSTRACT

Genes encoding wild type acetolactate synthase (ALS) and a sulfonylurea herbicide-resistant form of the enzyme, isolated from *Arabidopsis thaliana*, were expressed in transgenic *Nicotiana tabacum* plants under the control of their native promoters or of the highly active cauliflower mosaic virus 35S promoter. Expression of the wild type coding region from the 35S promoter resulted in a small, threefold increase in sulfonylurea tolerance above the levels measured in tissue expressing the native wild type gene. A much larger, 300-fold increase in herbicide tolerance was conferred by the mutant gene encoding a herbicide-resistant ALS. An additional 10-fold increase in tolerance was attained by expressing this coding region from the 35S promoter. The increase in both wild type and mutant gene expression directed by the 35S promoter resulted in over 25-fold higher levels of ALS messenger RNA in some transformants as compared with those expressing the native genes. However, ALS specific activity increased at most twofold, indicating that the amount of functional enzyme and messenger RNA are not correlated.

The ability to generate herbicide-tolerant plants or plant cell cultures by increasing expression of the wild-type target enzyme and/or by expressing a resistant form of the target enzyme has been demonstrated in several systems (18). These include phosphinothricin tolerance mediated through overexpression of glutamine synthase (26), glyphosate tolerance mediated through overexpression of EPSP[1] synthase (22) or through expression of a mutant *aroA* gene cloned from glyphosate-resistant *Salmonella typhimurium* (5, 25), and sulfonylurea/imidazolinone tolerance mediated through herbicide-resistant forms of ALS. Mutations in ALS (EC 4.1.3.18), the first common enzyme in the biosynthesis of the branched chain amino acids, were recovered in yeast (8), in bacteria (29), and in plants (4, 12) by selecting for resistance to sulfonylurea herbicides.

The gene encoding a sulfonylurea herbicide-resistant form of ALS isolated from *Arabidopsis thaliana* has a sequence identical to that for the wild-type gene of *Arabidopsis* except for a single base pair change that results in the substitution of serine for proline at the amino acid position 196 (11, 17). The analogous proline has been mutated to serine in a sulfonylurea herbicide-resistant form of yeast ALS (29) and to glutamine and alanine in the sulfonylurea-resistant tobacco ALS mutants SuRA C3 and SuRB S4-Hra, respectively (14). The mutant *Arabidopsis* ALS gene was shown to confer high levels of sulfonylurea resistance in transgenic tobacco plants (11).

Here we make use of the wild-type and mutant *Arabidopsis* ALS genes to compare sulfonylurea herbicide tolerance levels that can be obtained with normal and enhanced expression. Each gene, expressed from either its native promoter or from the highly active 35S promoter of CaMV (20), was used to generate transgenic tobacco plants. Tissue was assayed for levels of sensitive and resistant ALS enzyme activity, *Arabidopsis* ALS mRNA, and callus growth on the sulfonylurea herbicide chlorsulfuron. Progeny were assayed by seedling growth on chlorsulfuron.

MATERIALS AND METHODS

ALS Gene Transformation Vectors

The wild-type and sulfonylurea-resistant mutant *Arabidopsis* ALS genes were provided by B. Mazur and C. Somerville, respectively. Each was isolated on a 5.8 kb *Xba*I fragment (11, 17) that includes 2.5 kb of sequence 5′ to the translation start ATG and 1.3 kb of sequence 3′ to the translation stop codon. *Sal*I linkers were added to the *Xba*I ends and each gene was ligated, along with a 1.4 kb *Sal*I fragment containing the bacterial neomycin phosphotransferase I (nptI) gene, into the *Sal*I site of the intermediate vector pKNK (N Yadav, unpublished information) as shown in Figure 1A. This vector consists of pBR322 and a chimeric Nos promoter-nptII-Nos 3′ gene allowing kanamycin selection of transformed plant cells. The resulting plasmids pKAS and pKAR, containing the wild-type herbicide-sensitive and the mutant herbicide-resistant forms of the ALS gene, respectively, were introduced into *Agrobacterium tumefaciens* strain GV3850 (30) by three-way matings using *Escherichia coli* containing pRK2013 (7). *Agrobacterium* clones that had undergone recombination be-

[1] Abbreviations: EPSP, 5-enolpyruvylshikimate-3-phosphate; ALS, acetolactate synthase; CaMV, cauliflower mosaic virus; P:C:I, 50% phenol and 50% 24:1 chloroform:iso-amyl alcohol; kb, kilobase pair; bp, base pair; Nos, nopaline synthase; MS, Murashige-Skoog; SSC, standard saline citrate.

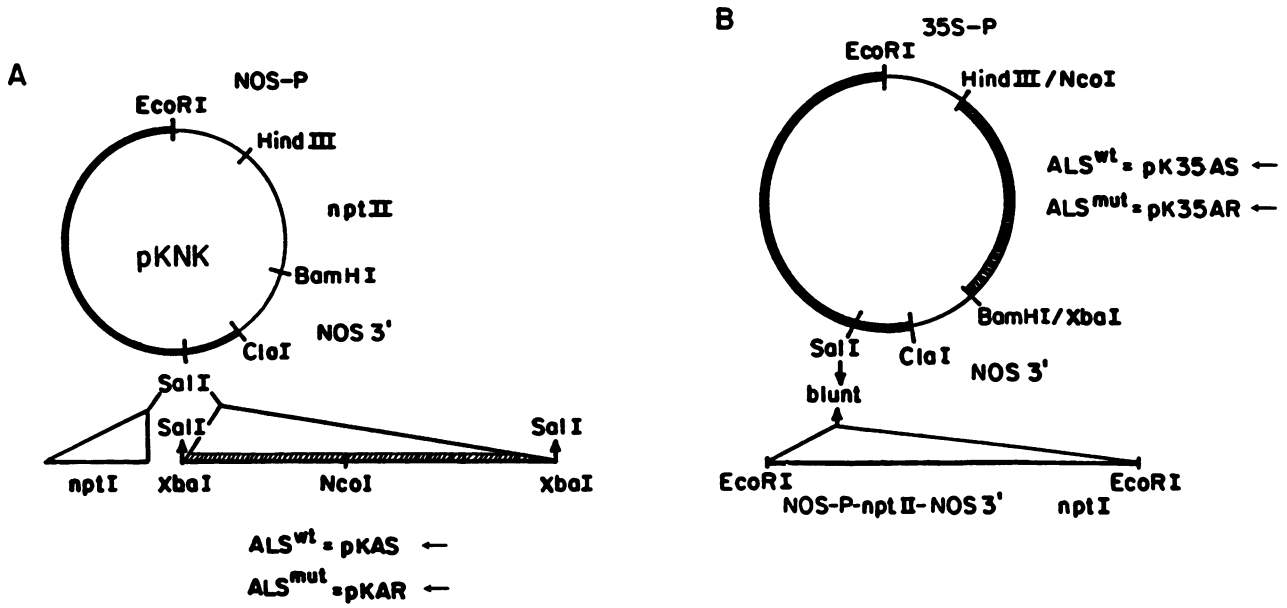

Figure 1. Diagrams of *Agrobacterium* transformation vectors. The heavy line portion of the circle is derived from pBR322. The hatched sections represent those derived from the ALS genes. A, Vector containing native wild type or mutant sulfonylurea herbicide-resistant *Arabidopsis* ALS gene. B, Vector containing chimeric gene constructions with the CaMV 35S promoter/leader substituted for the native promoter/leader of the wild type or sulfonylurea herbicide-resistant *Arabidopsis* ALS gene. Arrows mark the names of the final constructions.

tween pBR322 sequences in pKAS or pKAR and in the tDNA of the Ti-plasmid were selected on minimal plates containing M9 salts, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 0.4% sucrose, and 1 mg/mL kanamycin, and then verified by Southern blot analysis (24).

The native promoters of both the wild-type and mutant *Arabidopsis* ALS genes were replaced by the CaMV 35S promoter in the following manner. First the *Eco*RI-*Hind*III fragment of pKNK that contains the Nos promoter (Fig. 1A) was replaced by a 965 bp *Eco*RI-*Hind*III fragment containing the 35S promoter/leader (15) as shown in Figure 1B. In the resulting plasmid the *Hind*III-*Bam*HI fragment containing the nptII coding region was replaced with a 3.3 kb *Nco*I-*Xba*I fragment from either the wild-type or mutant ALS gene by blunt-end ligation. The *Nco*I site naturally occurs at the translation initiation site of the *Arabidopsis* ALS gene. Inserts in the correct orientation for ALS protein expression were identified by restriction analysis. The junction between the blunted *Hind*III and *Nco*I sites was sequenced to verify the presence of the translation initiation ATG in the proper reading frame. Then a 3.5 kb *Eco*RI fragment containing the bacterial nptI gene and the chimeric Nos-P-nptII-Nos 3′ gene was added as a blunt fragment into the *Sal*I site after the ends were filled using the Klenow enzyme, to serve as kanamycin selection markers. The resulting plasmids pK35AS and pK35AR containing the wild-type herbicide-sensitive and the mutant herbicide-resistant ALS genes, respectively, were recombined into the *A. tumefaciens* transfer DNA as described above.

Transformation

Leaf discs of *Nicotiana tabacum* cv Xanthi were inoculated with *A. tumefaciens* using a modification of the procedure of Horsch *et al.* (13). Discs were prepared from young, surface-sterilized leaves of 5- to 7-week-old chamber-grown plants using a sterile paper punch. They were dipped into a suspension of *A. tumefaciens* diluted to 1×10^8 cells/mL and then placed on a shoot regeneration medium consisting of MS salts (19), 100 mg/L i-inositol, 0.4 mg/L thiamine, 3% sucrose, 1 mg/L benzylaminopurine, 0.1 mg/L napthaleneacetic acid, and 0.8% agar, pH 5.8. No feeder layer was used. After 3 d of co-cultivation, the discs were placed on the same medium supplemented with 100 mg/L kanamycin and 500 mg/L cefotaxime. They were placed on fresh selective medium after 3 weeks. Regenerated shoots were excised from the discs 6 weeks after inoculation and placed on hormone-free rooting medium containing 100 mg/L kanamycin and 500 mg/L cefotaxime. Only shoots that rooted under selection were analyzed further.

Callus Assays

For a preliminary test of herbicide tolerance, several small leaves were excised from each kanamycin-resistant shoot, sliced into 2 to 3 mm pieces, and placed on callus induction medium that consisted of MS salts, 100 mg/L *i*-inositol, 0.4 mg/L thiamine, 3% sucrose, 1 mg/L napthaleneacetic acid, 0.2 mg/L benzylaminopurine, 0.8% agar, and 500 mg/L cefotaxime, pH 5.8, containing either 50 mg/L kanamycin, 10 ppb chlorsulfuron, or no selective agent. Callus formation was scored as plus or minus after 3 weeks of growth.

Quantitative callus growth tests were carried out as follows. Callus lines derived from transformed shoots were subcultured several times to generate quantities of relatively uniform tissue. Fifty milligrams of tissue was spread onto sterile double-paper filter discs (Whatman No. 1) and the discs were placed on the surface of the callus medium described above,

containing a series of chlorsulfuron concentrations. After a 2-week incubation, the callus tissue was scraped from each filter and weighed. Each treatment was replicated eight times. The mean growth for each treatment was expressed as the percent of the mean growth of untreated control tissue.

ALS Enzyme Assays

Plants were assayed 25 to 30 d after they were transplanted from culture plates to pots. Extracts were prepared from young, expanding leaves and assayed for ALS activity as described by Chaleff and Mauvais (3). The reaction product, acetoin, was quantified by measuring optical density at 530 nm (27) and protein concentrations were determined by the method of Bradford (2). For each extract, reactions containing either no herbicide or 100 ppb chlorsulfuron were sampled at different time points and the data were used to calculate the rate of product formation.

Construction and Labeling of RNA Probes

A 975-bp *Nco*I-*Bam*HI fragment from the 5′ end of the *Arabidopsis* ALS gene was subcloned into the pTZ19R vector (Pharmacia) between the *Sma*I and *Bam*HI sites. The filled-in *Nco*I end ligated to the *Sma*I-cut end regenerates the *Nco*I site, which was then used to linearize the DNA to produce a labeled RNA probe. RNA was produced as directed by the Promega Riboprobe RNA labeling system kit. An 820-bp *Bbv*I-*Bst*EII fragment from the 5′ end of the tobacco ALS gene, provided by Mazur *et al.* (17), was subcloned into pGEM 3 (Promega) between the *Bam*HI and *Sma*I sites by adding a *Bam*HI linker to the *Bbv*I end and filling in the *Bst*EII end with the Klenow enzyme. The DNA was linearized with *Xba*I to produce a labeled probe as described above.

RNA Preparation and Analysis

RNA was prepared according to the method of Hall *et al.* (10) with the following modifications. Five grams of leaf tissue were frozen in liquid nitrogen and ground to a fine powder. The powder was then added to 20 mL of extraction buffer (50 mM Tris, pH 9.0; 10 mM EDTA, pH 8.0; 2% SDS; and 200 μg/mL proteinase K) and placed in a 50°C water bath for 10 min. An equal volume of P:C:I was mixed in and the phases were then separated by centrifuging in 30 mL Corex tubes at 10,000 rpm for 10 min. The aqueous phase was removed and extracted a second time with P:C:I. Following the second extraction, the aqueous phase was precipitated by adding 1/10 volume of 3 M sodium acetate, pH 5.2, and an equal volume of isopropyl alcohol, and placing at −20°C overnight. Nucleic acids were pelleted by centrifuging at 10,000 rpm for 20 min. The pellet was dissolved in water at half the original lysis volume and an equal volume of 4 M LiCl was added. This was placed on ice for 1 h and then centrifuged at 10,000 rpm for 25 min. The supernatant was poured off, and the pellet that contained the RNA was resuspended in one-fourth the original lysis volume of water. Again, an equal volume of 4 M LiCl was added and the solution was treated as above. The pellet, now substantially free of DNA, was resuspended in water, extracted once with P:C:I, and precipitated with sodium acetate and 2 volumes of ethanol. The RNA pellet was resuspended in water and stored at −80°C. Polyadenylated RNA was isolated from the total RNA according to the procedure of Aviv and Leder (1).

Northern Hybridization

RNAs were denatured and separated by electrophoresis in formaldehyde-agarose gels according to the method of Rave *et al.* (21), with minor modifications. Agarose was dissolved in 260 mL of deionized water and 15 mL of 20× RNA buffer (0.4 M sodium tetraborate, 4 mM EDTA, pH 8.3) to a final concentration of 1%. When the agarose had cooled, 37% formaldehyde (Baker) was added to a final concentration of 6%. Before electrophoresis, RNA samples were denatured in 65% formamide, 20% formaldehyde, and 2× RNA buffer. The samples were heated to 65°C for 5 min and loaded directly onto the gel. Electrophoresis was carried out in 1× RNA buffer containing 3% formaldehyde at 25 V for 12 to 14 h. The gels were hydrolyzed in 50 mM NaOH and stained with ethidium bromide for 30 min. Next, the gels were neutralized in 0.2 M sodium acetate (pH 5.2) for two washes of 20 min each. Gels were photographed and then transferred overnight to Zeta-probe nylon filter paper (Bio-Rad) in a 10× SSC (3 M NaCl, 0.3 M trisodium citrate) transfer solution. The filters were baked under vacuum at 70°C for 2 h before prehybridization.

Filters were prehybridized at 65°C for 4 to 6 h in a solution containing 50% formamide; 5× SSC; 50 mM sodium phosphate, pH 6.5; 0.1% SDS; and 400 μg/mL sheared calf thymus DNA. Hybridizations were carried out in the same solution with the addition of 10% dextran sulfate (mol wt 500,000; Sigma) for 12 to 14 h at 65°C. The filters were washed at room temperature in 2× SSC, 0.2% SDS, and then at 65°C in 0.2× SSC, 0.2% SDS. RNA bands were visualized by autoradiography. Relative quantities of the *Arabidopsis* ALS mRNA in transgenic plants were assessed by comparing the hybridization signal in diluted RNA samples to the signal in the *Arabidopsis* RNA sample on Northern blots.

Seedling Tolerance Assays

Seeds were surface-sterilized by stirring for 30 min in 10% Clorox, 0.1% SDS and then rinsing three times with sterile, deionized water. They were then plated on a germination medium consisting of MS salts, 100 mg/L *i*-inositol, 0.4 mg/L thiamine, 3% sucrose, 0.8% agar, pH 5.8, containing chlorsulfuron concentrations from 3 ppb to 10 ppm. Each treatment comprised 50 to 100 seeds and was replicated twice. Seedling growth was scored after 3 weeks. Growth categories were: (a) true leaves/roots; (b) true leaves/no roots; (c) cotyledon expansion only; (d) no growth; and (e) no germination.

RESULTS

A chimeric gene construction was made to determine whether expressing the wild-type ALS coding region from a promoter known to have high activity could substantially increase the ALS mRNA level, ALS specific activity, and sulfonylurea herbicide resistance in transgenic plants. A par-

allel comparison was made using a gene that carries a single point mutation (11) and thereby encodes a herbicide-resistant form of ALS. *A. thaliana* ALS genes were used for expression studies in *N. tabacum* so that transcription from the introduced and endogenous ALS genes could be distinguished. Although the *Arabidopsis* and tobacco ALS genes (ALS^A and ALS^T, respectively) have a high degree of homology (17), separate probes can be made that distinguish between the *Arabidopsis* and tobacco ALS mRNAs under stringent conditions. We substituted the promoter/leader that directs expression of the 35S RNA of CaMV (20) for that of the *Arabidopsis* ALS gene using the naturally occurring *Nco*I site at the translation initiation ATG as described in "Materials and Methods" and shown in Figure 1. Transgenic tobacco plants carrying the chimeric 35S-P-ALS^A gene or the native ALS^A gene, both encoding wild-type ALS, were selected on kanamycin and are henceforth called 35AS and AS plants, respectively. Plants carrying the chimeric 35S-P-ALS^{AR} gene or the intact ALS^{AR} gene, both encoding the herbicide-resistant ALS, are henceforth called 35AR and AR plants, respectively.

As a preliminary assay of sulfonylurea herbicide tolerance levels conferred by the wild-type gene constructions, several small leaves were excised from each kanamycin-resistant shoot and tested for the ability to form callus either on kanamycin or on chlorsulfuron. Of 22 independent AS transformants that showed callus growth on kanamycin, none was able to form callus on 10 ppb chlorsulfuron. In contrast, 20 of 36 independent 35AS transformants formed callus on 10 ppb chlorsulfuron. This result demonstrates that replacing the native promoter of the wild-type ALS gene with the CaMV 35S promoter can produce increased tolerance to a low level of sulfonylurea herbicide.

To establish that the 35S promoter directed higher levels of gene expression than the native ALS promoter, RNA was prepared from individual transgenic plants and assayed using Northern blot hybridization. A 5′ fragment from the *Arabidopsis* ALS gene used as a probe detected the *Arabidopsis,* but not the endogenous tobacco, ALS mRNA. This specificity is shown in Figure 2, A and B, in which no hybridization to total or to polyadenylated RNA from wild-type tobacco, or to total RNA from tobacco plants transformed with the kanamycin resistance vector lacking an ALS gene, can be seen. The level of ALS^A mRNA expression directed by the 35S promoter varied greatly between independent transformants. Since we were interested in determining the maximal effects of increased expression on enzyme activity and herbicide tolerance, instead of choosing representative 35AS and 35AR plants for further analysis, we chose only those plants that produced the highest levels of ALS^A or ALS^{AR} mRNA. Figure 2A shows the levels of ALS^A mRNA found when expression of the wild-type gene was from the native promoter (AS plants 4 and 13), and the levels present in the two plants with the highest levels of expression of the 35AS chimeric gene (35AS plants 37 and 38B). 35AS plant 37 had over 25-fold more ALS^A mRNA than did AS plant 4. The series of smaller hybridizing fragments in lanes 8 and 9 may be degradation products of the highly abundant ALS^A mRNA found in the 35AS transformants. Results using a probe specific for tobacco ALS on similar Northern blots showed that the endogenous ALS mRNA level remained constant in all transformants (data not shown). Figure 2B demonstrates that in plants receiving the gene encoding the herbicide-resistant form of ALS, expression from the 35S promoter can also result in over 25-fold more ALS^{AR} mRNA (35AR plants 2 and 9) than expression from the native promoter (AR plants 7, 9B, and 25).

The effects, on a functional level, of high ALS^A and ALS^{AR} mRNA expression were analyzed by ALS enzyme assays and quantitative callus growth tests. Since ALS enzyme activity varied with plant age (data not shown), comparisons were only made between plants assayed at the same stage of growth. The extracts assayed for enzyme activity were prepared from half of the leaf used to prepare the RNA, so that there was no physiologic difference in the plant material used in the two assays. The results of the enzyme activity assays indicated that gene overexpression led to no more than a twofold increase in ALS specific activity (values given in Fig. 2 legend). Thus, the specific activity of ALS does not correlate with the level of ALS mRNA expressed from the 35S promoter, suggesting that there is some type of post-transcriptional regulation of the total amount of active ALS enzyme found in plant leaves.

In plants carrying the gene encoding the herbicide-resistant form of ALS it is possible to distinguish the activity contributed by the introduced gene from that contributed by the endogenous genes. A concentration of 100 ppb chlorsulfuron inhibits the wild-type tobacco ALS enzyme by 98%, whereas the *Arabidopsis*-resistant ALS enzyme remains fully active (12). Figure 2C compares ALS activity in the presence of 100 ppb chlorsulfuron to total ALS activity measured in the absence of herbicide. In plants with the resistant gene expressed from the native promoter, 40 to 60% of the total ALS activity is derived from the introduced gene. However, in the plants in which the resistant gene is expressed from the 35S promoter, 85 to 90% of activity comes from the introduced gene. Thus the resistant enzyme displaces most of the sensitive enzyme.

Quantitative callus growth tests were carried out using tissue derived from the same transformants (Fig. 3). AS plants were virtually intolerant to 1 ppb chlorsulfuron, as was the case for the control transformant. The most tolerant 35AS plants showed a level of callus growth on 1 ppb equivalent to that which the AS plants showed on 0.3 ppb, indicating a threefold increased level of tolerance to chlorsulfuron. This small increase in tolerance to chlorsulfuron in the growth test does not correlate with the large increase in ALS^A mRNA level observed, but does correlate with the small increase in ALS specific activity. As shown by the examples in Figure 3B, calli from several AR plants generally grew as well on 30 ppb chlorsulfuron as callus from AS plants grew on 0.1 ppb, demonstrating a 300-fold increased tolerance to chlorsulfuron. The substitution of the 35S promoter, in the 35S-P-ALS^{AR} chimeric gene, raised the tolerance another 10-fold allowing a comparable amount of callus growth on 300-ppb chlorsulfuron.

Cosegregation of increased herbicide tolerance and increased ALS^A mRNA levels was demonstrated in progeny derived from self-pollination of 35AS plant 38B. In a quantitative callus growth test on leaf material from six randomly chosen progeny, four showed growth on 1 ppb chlorsufuron

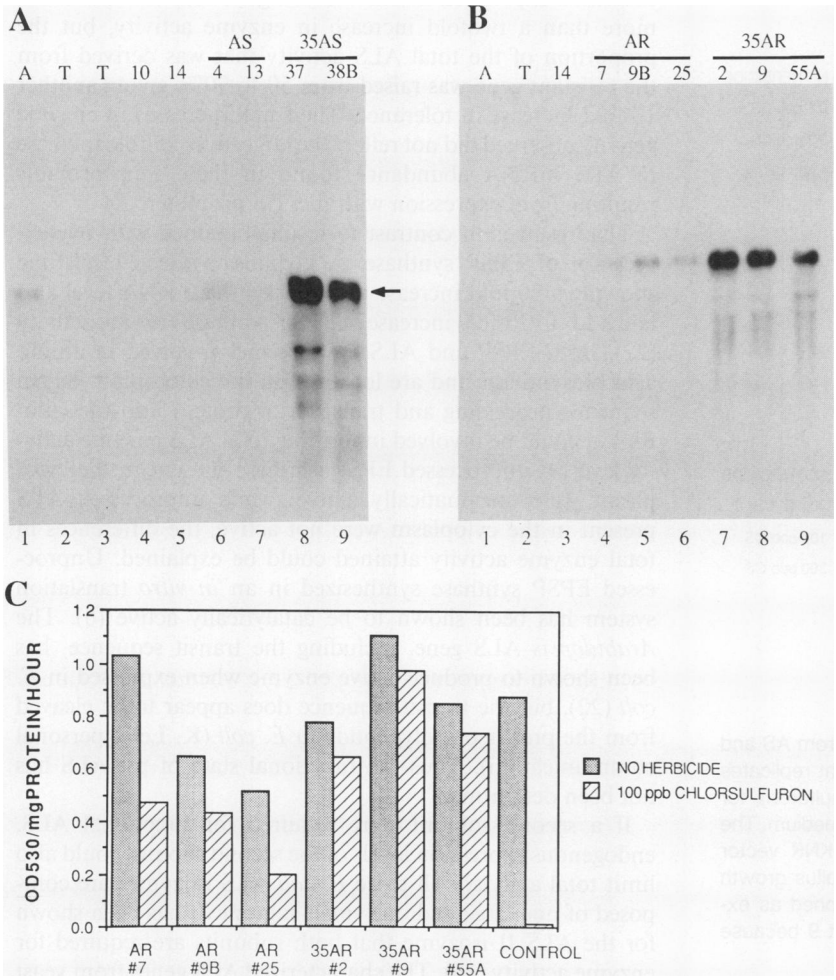

Figure 2. A, Northern blot analysis of RNA isolated from AS and 35AS tobacco plants. Each lane contains 20 μg of total RNA except lane 2, which contains 5 μg of polyadenylated RNA. A 975-bp 5′ fragment of the *Arabidopsis* ALS gene was used as the hybridization probe. Lane 1, RNA from *A. thaliana*; lanes 2 and 3, RNA from nontransformed tobacco plants; lanes 4 and 5, RNA from two different plants transformed with the pKNK vector containing no *Arabidopsis* ALS gene; lanes 6 and 7, RNA from two different AS plants; lanes 8 and 9, RNA from two different 35AS plants. All lanes are from the same Northern blot and the same film exposure. The number given to each transformed plant is listed above each lane. The arrow shows the band representing the full-length ALS mRNA (approximately 2.3 kb), as determined by its comigration with the hybridizing band in the lane containing *Arabidopsis* RNA. The specific activities for ALS in each plant expressed as change in OD530/mg protein/h are: AS#4, 0.64; AS#13, 0.70; 35AS#37, 0.82; and 35AS#38B, 1.43. B, Northern blot hybridization analysis of RNA isolated from AR and 35AR plants. Lane 1, RNA from *A. thaliana*; lane 2, RNA from a nontransformed tobacco plant; lane 3, RNA from a plant transformed with the pKNK vector containing no *Arabidopsis* ALS gene; lanes 4, 5, and 6, RNA from three different AR plants; lanes 7, 8, and 9, RNA from three different 35AR plants. The specific activities for ALS in each plant expressed as change in OD530/mg protein/h are: AR#7, 1.0; AR#9B, 0.65; AR#25, 0.52; 35AR#2, 0.78; 35AR#9, 1.1; 35AR#55A, 0.85. C, Chlorsulfuron-resistant ALS activity in AR and 35AR transformants. The ALS specific activity assayed in the presence of 100 ppb chlorsulfuron is graphed adjacent to the total ALS specific activity for each plant, showing the proportion of resistant activity. Specific activities are expressed as change in OD530/mg protein/h.

and two did not. The four that grew all had high levels of ALS^A mRNA equivalent to that of the parent plant 38B, whereas the two that did not grow had no detectable ALS^A mRNA (data not shown). This experiment confirms that increased expression of the wild-type *Arabidopsis* ALS gene causes an increase in tolerance to chlorsulfuron.

Analysis of Seedling Tolerance to Chlorsulfuron

In order to determine whether the differences in callus tolerance found in the transformants would also be maintained at the whole plant level, seeds derived from self-pollinations were germinated on different concentrations of chlorsulfuron. Untransformed tobacco seeds failed to form roots and true leaves on concentrations higher than 10 ppb. We have therefore used the formation of these organs as our definition of tolerance.

Seeds derived from plants transformed with the wild-type *Arabidopsis* ALS gene, regulated by either its native promoter or by the CaMV 35S promoter, did not differ in tolerance to chlorsulfuron from seeds derived from a vector-transformed control plant. This was not unexpected, given the small differences in tolerance seen in the sensitive callus assay.

Progeny of plants transformed with the *Arabidopsis* gene encoding the sulfonylurea herbicide-resistant ALS were tolerant to chlorsulfuron concentrations as high as 3 ppm (Fig. 4), a 300-fold increase in tolerance above controls. This difference was consistent with the difference seen in the callus growth assay. In each of the three lines tested, approximately 75% of the seedlings formed true leaves at 3 ppm, whereas approximately 25% died shortly after germination. This suggests that the introduced ALS gene is segregating as a single genetic locus in these lines. However, Figure 4 demonstrates that the expression of full tolerance, leaf and root formation, varies among transformants and is dependent upon herbicide concentration. For example, AR plant 7 segregated 73% with leaves/roots and 27% dead at 1 ppm, and 32% with leaves/roots, 40% with leaves only, and 28% dead at 3 ppm. These results indicate expression of the introduced ALS gene as a semi-dominant marker.

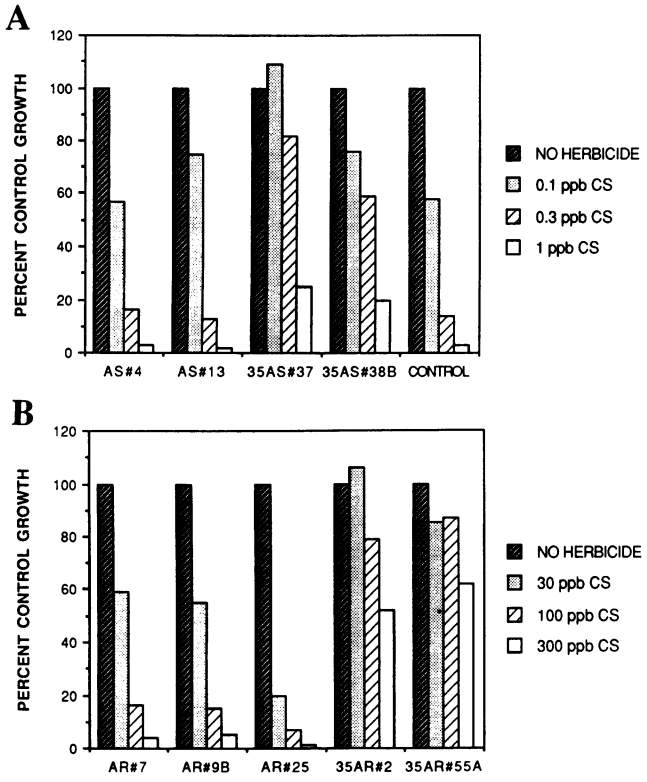

Figure 3. A, Quantitative callus growth tests of tissue from AS and 35AS plants. The mean of the amount of growth in eight replicates is graphed as the percent of control growth (no chlorsulfuron), for each different concentration of chlorsulfuron (CS) in the medium. The control is callus from a plant transformed with the pKNK vector containing no *Arabidopsis* ALS gene. B, Quantitative callus growth tests of tissue from AR and 35AR transformants. Graphed as explained for A. No callus growth test was done for plant 9 because adequate amounts of callus tissue were not obtained.

Seedlings derived from plants transformed with the resistant ALS gene regulated by the CaMV 35S promoter were tolerant to chlorsulfuron concentrations as high as 10 ppm (Fig. 4), a 1000-fold increase in tolerance above controls and a threefold increase above the most tolerant AR plants. This increase is compatible with that seen in the callus growth assay. These lines also demonstrated segregation of tolerance consistent with the insertion of a semi-dominant gene at a single locus.

DISCUSSION

We have compared the levels of sulfonylurea herbicide tolerance that can be achieved in transgenic plants by increased expression of a wild-type ALS gene, by normal expression of a resistant ALS gene, and by increased expression of the resistant gene. We have found that increased expression of the wild-type ALS gene is not sufficient to confer useful levels of herbicide resistance in tobacco. Increased expression of the wild-type *Arabidopsis* gene in tobacco resulted in no more than a twofold increase in ALS enzyme activity, which could support only a threefold increase in herbicide tolerance. However, the mutant *Arabidopsis* ALS gene, having only the one amino acid change of position-196 proline to serine, conferred a 300-fold increase in resistance to chlorsulfuron. Increased expression of the mutant gene resulted in still no more than a twofold increase in enzyme activity, but the proportion of the total ALS activity that was derived from the resistant gene was raised from 50 to 90%, giving another 10-fold increase in tolerance. The small increases in enzyme activity observed did not reflect the large, over 25-fold increase in ALS mRNA abundance found in these transformants resulting from expression with the 35S promoter.

This result is in contrast to results obtained with overexpression of EPSP synthase. A glyphosate-selected cell line showing a 20-fold increase in EPSP synthase RNA level also had a 15- to 20-fold increase in EPSP synthase enzyme activity (22). Both EPSP and ALS are enzymes involved in amino acid biosynthesis and are localized in the chloroplast. Signal sequence processing and transport of protein into the chloroplast could be involved in limiting total ALS enzyme activity level. If unprocessed EPSP synthase present in the cytoplasm were enzymatically active, while unprocessed ALS present in the cytoplasm were not active, the differences in total enzyme activity attained could be explained. Unprocessed EPSP synthase synthesized in an *in vitro* translation system has been shown to be catalytically active (6). The *Arabidopsis* ALS gene, including the transit sequence, has been shown to produce active enzyme when expressed in *E. coli* (22), but the transit sequence does appear to be cleaved from the precursor polypeptide in *E. coli* (K. Leto, personal communication). Thus the functional state of pre-ALS has not been determined.

If a second subunit were required for functional ALS, endogenous expression levels of the second subunit could also limit total activity. All three *E. coli* ALS isozymes are composed of one large and one small subunit. It has been shown for the ALS II isozyme that both subunits are required for enzyme activity (16). The characterized ALS genes from yeast and plants correspond in sequence to the large subunit of the *E. coli* enzyme (8, 17). However, there is evidence against the existence of a second subunit in yeast and plant ALS. In yeast,

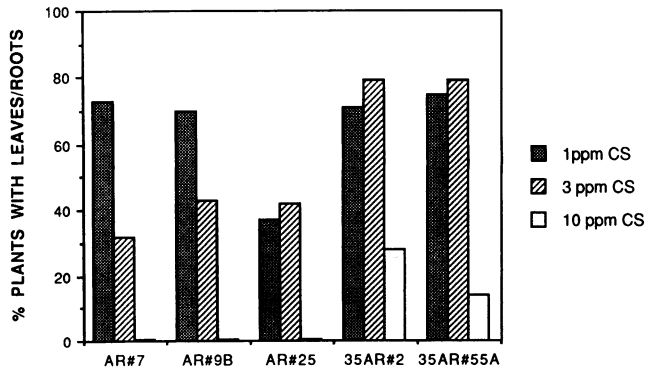

Figure 4. Tolerance of progeny of AR and 35AR plants to chlorsulfuron. The number of germinated seedlings that developed true leaves and roots is presented as the percent of total germinating seeds placed on each concentration of chlorsulfuron (CS). The number corresponding to the parent plant is listed below each set of progeny data.

when the "large subunit" ALS is expressed on a multicopy plasmid the enzyme activity increases fourfold (9), and when it is expressed from the induced Gal10 promoter the activity increases 10-fold (C Falco, personal communication). Whether these increases in activity correlated with increases in ALS mRNA levels was not determined. This yeast data could be misleading due to the pre-existence of an excess of a "small subunit" in the cells or to the regulation of the quantity of a "small subunit" by the amount of "large subunit" in the cells. As mentioned above, the *Arabidopsis* ALS "large subunit" gene can be expressed in *E. coli* to produce active enzyme, suggesting that a small subunit is not required (23). These explanations assume that the amount of ALS mRNA present in the cell reflects the amount of ALS protein. It is also possible that some block exists at the level of translation, or of protein stability. The untransported, unprocessed ALS polypeptide could be rapidly turned over in the cytoplasm. Williamson *et al.* (28) found a much reduced level of protein as compared with its mRNA when a 19-kD zein coding region was expressed from a β-phaseolin gene promoter in transgenic petunia. Both of these systems involve processing, transport, and localization, processes that may be inefficient in a heterologous plant system.

An unexpected finding in this study is that expression of a high level of ALS mRNA in transgenic tobacco plants does not result in a correspondingly high level of the encoded enzyme activity. However, by using the 35S promoter, instead of raising the level of total ALS enzyme activity as we had expected, we were able to raise the proportion of total ALS activity that was derived from the resistant *Arabidopsis* ALS gene from 50 to 90%, thereby increasing the chlorsulfuron resistance an additional 10-fold. This increase could provide the difference between an inadequate and a usable level of resistance to a herbicide in field situations, in selection schemes in tissue culture, or in other applications.

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LITERATURE CITED

1. **Aviv H, Leder P** (1972) Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc Natl Acad Sci USA **69:** 1408–1412
2. **Bradford MM** (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. Anal Biochem **72:** 248–254
3. **Chaleff RC, Mauvais CJ** (1984) Acetolactate synthase is the site of action of two sulfonylurea herbicides in higher plants. Science **224:** 1443–1445
4. **Chaleff RC, Ray TB** (1984) Herbicide-resistant mutants from tobacco cell cultures. Science **223:** 1148–1151
5. **Comai L, Facciotti D, Hiatt WR, Thompson G, Rose RE, Stalker DM** (1985) Expression in plants of a mutant *aro*A gene from *Salmonella typhimurium* confers tolerance to glyphosate. Nature **317:** 741–744
6. **Della-Cioppa G, Bauer SC, Klein BK, Shah DM, Fraley RT, Kishore GM** (1986) Translocation of the precursor of 5-enolpyruvylshikimate-3-phosphate synthase into chloroplasts of higher plants *in vitro*. Proc Natl Acad Sci USA **83:** 6873–6877
7. **Ditta G, Stanfield S, Corbin D, Helinski D** (1980) Broad host range DNA cloning system for Gram-negative bacteria. Construction of a gene bank of *Rhizobium meliloti*. Proc Natl Acad Sci USA **77:** 7347–7351
8. **Falco SC, Dumas KS** (1985) Genetic analysis of mutants of *Saccharomyces cerevisiae* resistant to the herbicide sulfometuron methyl. Genetics **109:** 21–35
9. **Falco SC, Dumas KS, Livak K** (1985) Nucleotide sequence of the yeast ILV2 gene which encodes acetolactate synthase. Nucleic Acids Res **13:** 4011–4027
10. **Hall TL, Ma Y, Buchbinder BU, Pyne JW, Son SM, Bliss FA** (1978) Messenger for G1 protein of French bean seeds. Cell-free translation and product characterization. Proc Natl Acad Sci USA **75:** 3196–3200
11. **Haughn GW, Smith J, Mazur B, Somerville C** (1988) Transformation with a mutant *Arabidopsis* acetolactate synthase gene renders tobacco resistant to sulfonylurea herbicides. Mol Gen Genet **211:** 266–271
12. **Haughn GW, Somerville C** (1986) Sulfonylurea-resistant mutants of *Arabidopsis thaliana*. Mol Gen Genet **204:** 430–434
13. **Horsch RB, Fry JE, Hoffman NL, Wallroth M, Eicholtz D, Rogers SG, Fraley RT** (1985) A simple and general method for transferring genes into plants. Science **227:** 1229–1231
14. **Lee KY, Townsend J, Tepperman J, Black M, Chui CF, Mazur B, Dunsmuir P, Bedbrook J** (1988) The molecular basis of sulfonylurea herbicide resistance in tobacco. EMBO J **7,5:** 1241–1248
15. **Lin W, Odell JT, Schreiner RM** (1987) Soybean protoplast culture and direct gene uptake and expression by cultured soybean protoplasts. Plant Physiol **84:** 856–861
16. **Lu M-F, Umbarger HE** (1987) Effects of deletion and insertion mutations in the *ilv*M gene of *Escherichia coli*. J Bacteriol **169:** 600–604
17. **Mazur BJ, Chui CF, Smith JK** (1987) Isolation and characterization of plant genes for acetolactate synthase, the target enzyme for two classes of herbicides. Plant Physiol **85:** 1110–1117
18. **Mazur BJ, Falco SC** (1989) The development of herbicide resistant crops. Ann Rev Plant Physiol Plant Mol Biol **40:** 441–470
19. **Murashige T, Skoog F** (1962) A revised medium for rapid growth and bio-assay with tobacco tissue cultures. Physiol Plant **15:** 473–497
20. **Odell JT, Nagy F, Chua NH** (1985) Identification of sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature **313:** 810–812
21. **Rave N, Crvenjakov R, Boedtker H** (1979) Identification of procollagen mRNAs transferred to diazobenzyloxymethyl paper from formaldehyde agarose gels. Nucleic Acids Res **6:** 3559–3569
22. **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
23. **Smith JK, Schloss JV, Mazur BJ** (1989) Functional expression of plant acetolactate synthase genes in *Eschericia coli*. Proc Natl Acad Sci USA **86:** 4179–4183
24. **Southern EM** (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol **98:** 503–517

25. **Stalker D, Hiatt W, Comai L** (1985) A single amino acid substitution in the enzyme 5-enolpyruvylshikimate-3-phosphate synthase confers resistance to the herbicide glyphosate. J Biol Chem **260:** 4724–4728
26. **Tischer E, DasSarma S, Goodman HM** (1986) Nucleotide sequence of an alfalfa glutamine synthase gene. Mol Gen Genet **203:** 221–229
27. **Westerfield WW** (1945) A colorimetric determination of blood acetoin. J Biol Chem **161:** 495–502
28. **Wiliamson JD, Galili G, Larkins BA, Gelvin SB** (1988) The synthesis of a 19 kilodalton zein protein in transgenic petunia plants. Plant Physiol **88:** 1002–1007
29. **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
30. **Zambryski P, Joos H, Genetello C, Leemans J, Van Montagu M, Schell J** (1983) Ti-plasmid vector for the introduction of DNA into plant cells without altering their normal regeneration capacity. EMBO J **2:** 2143–2150