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# The molecular basis of sulfonylurea herbicide resistance in tobacco

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**The enzyme acetolactate synthase (ALS) is the target enzyme for the sulfonylurea and imidazolinone herbicides. We describe the isolation and characterization of the ALS genes from two herbicide-resistant mutants, C3 and S4-Hra, of *Nicotiana tabacum*. There are two distinct ALS genes in tobacco which are 0.7% divergent at the amino acid sequence level. The C3 mutant has a single Pro-Gln replacement at amino acid 196 in one ALS gene. This gene is termed the class I gene and is equivalent to the SuRA locus. The S4-Hra mutant has two amino acid changes in the other ALS gene. This gene is termed the class II gene or the SuRB locus. The S4-Hra mutant includes a Pro-Ala substitution at amino acid 196 and a Trp-Leu substitution at amino acid 573. Gene reintroduction experiments have confirmed that these amino acid substitutions are responsible for the herbicide resistance phenotypes. Transgenic plants carrying these genes are highly resistant to sulfonylurea herbicide applications.**

*Key words:* acetolactate synthase/herbicide resistance/mutation/*Nicotiana tabacum*/sulfonylurea

## Introduction

Acetolactate synthase (ALS; EC 4.1.3.18) catalyzes the initial common step in the biosynthesis of the branched chain amino acids valine, leucine and isoleucine. This enzyme is the primary target for two classes of herbicides: the sulfonylureas (LaRossa and Schloss, 1984) and the imidazolinones (Shaner *et al.*, 1984). Mutant forms of ALS genes in yeast (Falco and Dumas, 1985), in bacteria (Yadav *et al.*, 1986) and in plants (Chaleff and Ray, 1984; Chaleff and Mauvais, 1984; Haughn and Somerville, 1986) have been identified by selecting for resistance to these herbicides. Using this approach six mutants in *Nicotiana tabacum* were isolated by Chaleff and Ray (1984) by selection for tissue culture lines resistant to the sulfonylurea herbicides chlorsulfuron (Glean® ) and sulfometuron methyl (Oust® ). Genetic analyses of these six mutants defined two unlinked loci in *N.tabacum*, designated SuRA and SuRB. The two loci were defined by the independently isolated mutants C3 (SuRA) and S4 (SuRB). Subsequent selection for increased resistance in the S4 line led to the identification of a third type of mutant, Hra, which was linked to the S4 mutation at the SuRB locus but unlinked to the C3 mutation (Chaleff *et al.*, 1986). Biochemical analyses showed that the mutations cosegregated with herbicide-insensitive ALS activity and that resistance was due to the production of an altered form of the ALS enzyme which was less sensitive to the herbicide, rather than due to the overproduction of a normal enzyme (Chaleff and Mauvais, 1984). These data suggest that there are at least two unlinked ALS genes in *N.tabacum* (Chaleff and Mauvais, 1984). In a similar study in *Arabidopsis*, a mutation in a single ALS gene has been identified which confers chlorsulfuron resistance (Haughn and Somerville, 1986).

Here we present the isolation and molecular characterization of ALS genes from mutant *N.tabacum* lines which specify herbicide-resistant forms of the ALS enzyme. The molecularly characterized ALS genes are assigned to the genetic loci which define the sulfonylurea resistance. The mutant ALS genes confer herbicide resistance when transferred to sensitive lines of tobacco and the transgenic plants so derived are highly resistant to herbicide applications. We show that the resistant phenotypes are the consequence of mutations in the coding regions of the ALS gene, and are not due to duplications or amplifications of the genes or to changes in the regulatory regions surrounding the genes. This study provides a molecular basis for designing herbicide-resistant plant varieties and makes possible further research in plant cell somatic genetics by providing a new selectable marker for plant transformation experiments.

## Results and discussion

### *There are two distinct genes for acetolactate synthase in N.tabacum*

Our first objective was to determine if there were any gross structural differences between the ALS genes of wild-type *N.tabacum* and the herbicide-resistant C3 and S4-Hra mutant lines. Mazur *et al.* (1987) have described the isolation of a gene specifying ALS from *N.tabacum*. Figure 1A illustrates the results of hybridizations to restriction-enzyme-digested tobacco DNA using probes derived from the 5′ and 3′ halves of this cloned ALS gene (shown in Figure 2). In *N.tabacum* the 5′ probe hybridizes to 7-kb and 1.3-kb *Eco*RI bands and the 3′ probe hybridizes to 7-kb and 5.6-kb *Eco*RI bands (lane A). Both 5′ and 3′ probes hybridize to 6.2-kb and 4.7-kb *Nco*I bands (lane D) and both probes also hybridize to 10-kb and 8.3-kb *Spe*I bands of *N.tabacum* DNA (data not shown). The hybridization of both the 5′ and 3′ probes to two sets of fragments suggests that there are two distinct restriction enzyme polymorphs of the ALS gene in the tobacco genome, and that there are at least two distinct genes encoding ALS in this species. This is consistent with the genetic data from the mutant C3 and S4-Hra lines which indicate that there are three distinct mutations occurring at two genetically unlinked loci, SuRA and SuRB respectively.

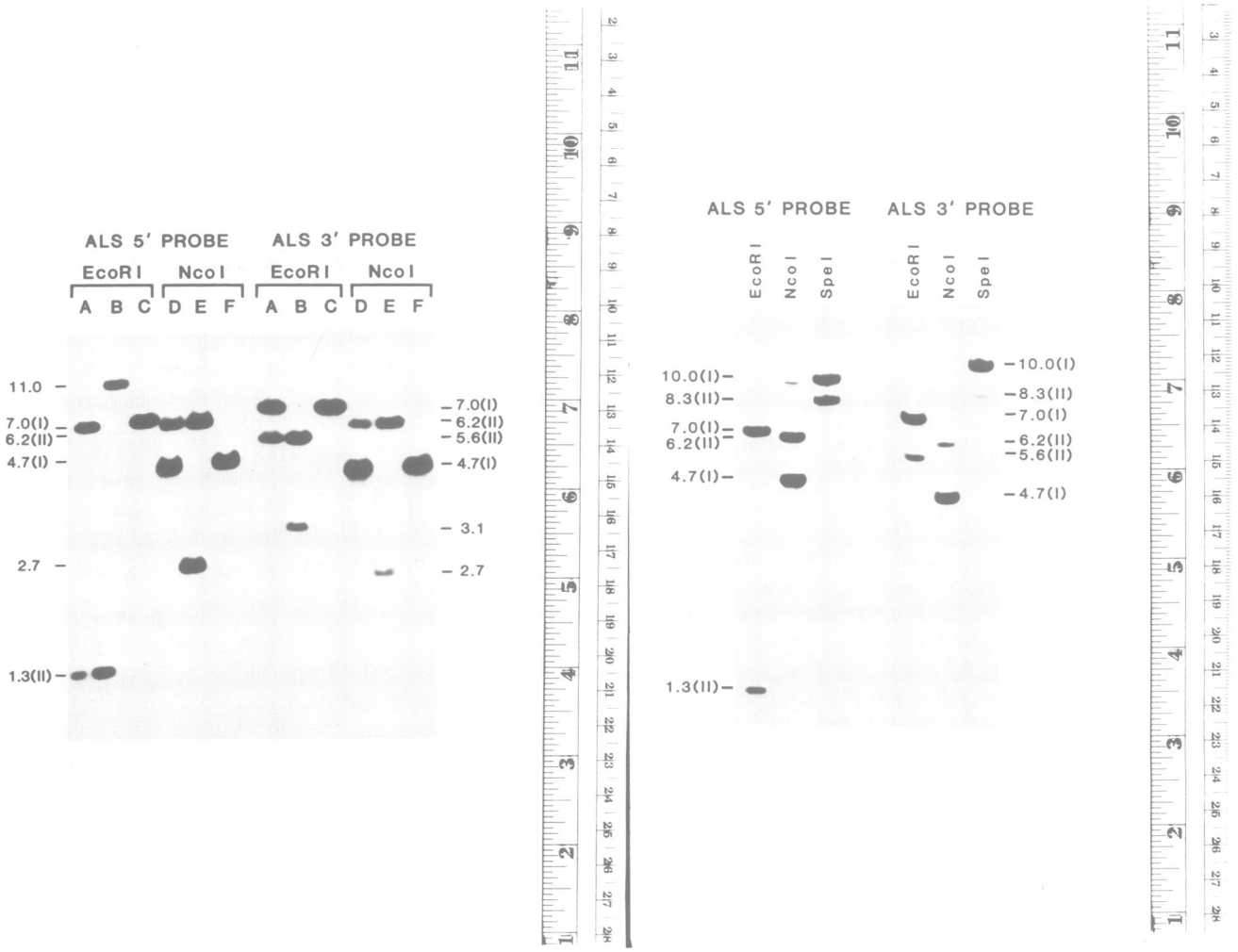



**Fig. 1.** (**A**) ALS gene hybridizations to genomic DNA of *Nicotiana* sp. Genomic DNA from *N.tabacum* (**lanes A** and **D**), *N.tomentosiformis* (**lanes B** and **E**) and *N.sylvestris* (**lanes C** and **F**) was digested with *Eco*RI or *Nco*I, subjected to electrophoresis through a 0.8% agarose gel, transferred to a nitrocellulose filter and hybridized to ALS gene 5′ and 3′ specific probes. Fragment sizes in kb are shown; the assignment of bands to class I or II ALS genes, as described in the text, is given in parentheses. (**B**) Genomic DNA from the S4-Hra mutant tobacco line was digested with *Eco*RI, *Nco*I or *Spe*I. The digested DNA was treated as in (A).

Hybridization of the 5′ and 3′ ALS gene probes to genomic DNA from the mutant S4-Hra (Figure 1B) and C3 lines gave the identical hybridization patterns as found for wild-type DNA; there was no indication of rearrangement, duplication or amplification of the ALS gene in these mutant lines.

In order to define the molecular basis of the herbicide resistance mutations we isolated and sequenced representatives of the two gene types from homozygous mutant plants of the C3 and S4-Hra genotypes. It was our expectation based upon genetic data (Chaleff *et al.*, 1986) that the mutations to resistance would occur in different genes, and therefore that each mutant line would contain a wild-type form and a mutant form of the ALS gene.

Genomic libraries were prepared from S4-Hra and C3 lines; 600 000 phage clones from each library were screened separately for homology to both the 5′and the 3′ ALS gene probes. Eleven hybridizing clones were obtained from these two libraries. Figure 2 illustrates the physical cleavage maps of these 11 clones together with the approximate position of the ALS coding region. The clones fall into two classes defined by their restriction enzyme cleavage maps: class I is typified by ALS phage 1 and class II is typified by ALS phage 3. The class I genes overlap the 7-kb *Eco*RI, the 4.7-kb *Nco*I and the 10-kb *Spe*I fragments observed in the genomic hybridization experiments. The class II genes overlap the 1.3-kb and 5.6-kb *Eco*RI, the 6.2-kb *Nco*I and the 8.3-kb *Spe*I fragments. Exhaustive restriction enzyme analyses showed no differences in physical maps between the independently isolated clones for each gene class. These results, together with the genomic hybridization data, indicate that there are only two classes of ALS genes in *N.tabacum*. Further, for the restriction endonucleases we have used, no restriction polymorphism identifies resistant or sensitive alleles. The class I and class II genes defined by this analysis probably correlate with the two independent genetic loci defined by the C3 and S4-Hra mutations.

### *The two ALS gene classes in N.tabacum are derived from the separate progenitor species*

*N.tabacum* is an allotetraploid species which is believed to have arisen from the hybridization of the diploid species *Nicotiana tomentosiformis* and *Nicotiana sylvestris* (Smith, 1975). Either parental species could have contributed both classes of ALS genes to *N.tabacum*, or alternatively each parental species may have contributed only one of the two classes of ALS gene. To distinguish the sources of the ALS

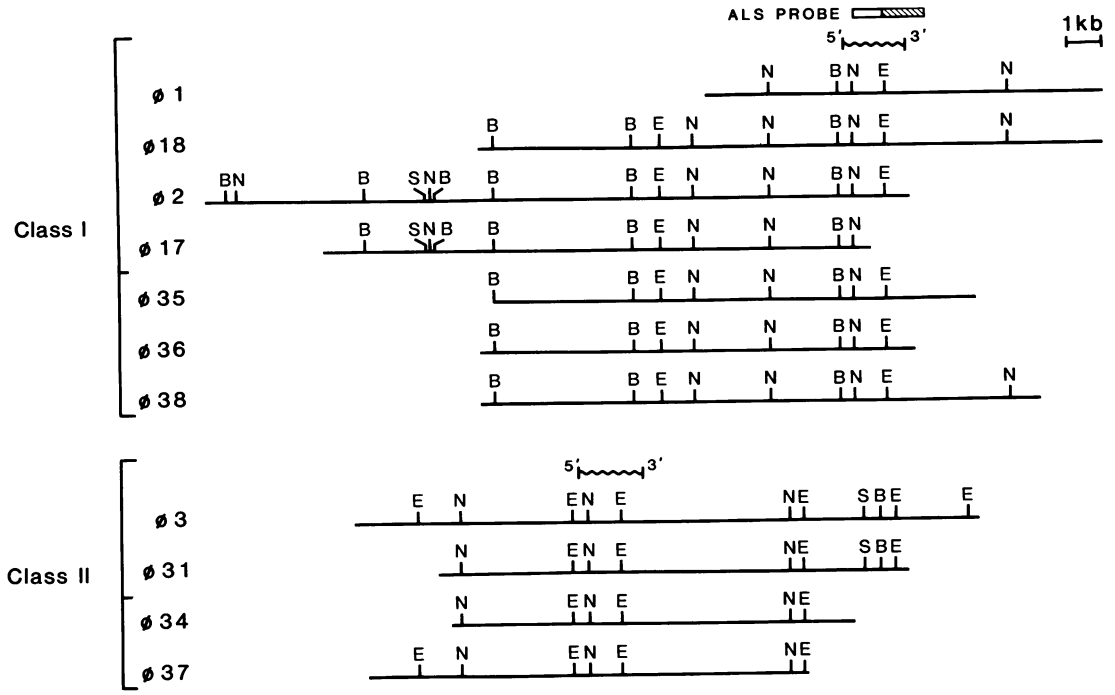



**Fig. 2.** Restriction endonuclease maps of phage clones carrying tobacco ALS genes. EMBL3 phage bearing genomic DNA with tobacco ALS genes were digested with restriction enzymes. Phages 1, 18, 2, 17 and 3 were isolated from an S4-Hra library. Phages 35, 36, 38, 31, 34 and 37 were isolated from a C3 library. The restriction maps of these phage define two distinct classes of ALS genes, as indicated in the figure. The positions and orientations of the ALS genes are shown with a wavy line. The locations of the 5′ specific ☐ and 3′ specific ▨ ALS gene probes are shown. Restriction sites are abbreviated as follows: B, *Bam*HI; E, *Eco*RI; N, *Nco*I; S, *Sal*I.

genes in *N.tabacum*, we analyzed the structure of these genes in the genomes of the progenitors *N.tomentosiformis* and *N.sylvestris*. As shown in Figure 1A, the class I ALS gene of *N.tabacum*, typified by the 7.0-kb *Eco*RI and 4.7-kb *Nco*I fragments (lanes A and D), is contributed by *N.sylvestris* (lanes C and F) and the class II ALS gene of *N.tabacum*, characterized by the 1.3-kb *Eco*RI and 6.2-kb *Nco*I fragments, is contributed by *N.tomentosiformis* (lanes B and E). Additional 11-kb and 3.1-kb *Eco*RI and 2.7-kb *Nco*I fragments which are not found in *N.tabacum* DNA hybridize in *N.tomentosiformis* DNA. We have not characterized this hybridization further although we assume that it is indicative of an additional ALS gene in *N.tomentosiformis*. These genomic hybridizations show that the two different classes of ALS genes in *N.tabacum* are derived from the separate progenitor species and imply that there are two (rather than four) separate genes for ALS in *N.tabacum*.

***Mutant ALS genes can be detected by gene transfer***

In order to establish a relationship between the ALS genes which we had cloned and the genetic loci defining herbicide resistance, we transferred the cloned ALS genes to sensitive tobacco cells and tested the resulting transgenic tissue for a herbicide resistance phenotype. We transferred two examples of a class I ALS gene derived from the C3 mutant library (phages 35 and 38), two examples of a class I gene from the S4-Hra mutant library (phages 1 and 18) and one example of a class II gene derived from a C3 mutant library (phage 31) and the S4-Hra mutant library (phage 3).

The genomic fragments containing the representative class I and class II genes from each mutant were first subcloned into the plant transformation vector pAGS135 (C.Dean *et al.*, in preparation) which confers resistance to the drug kanamycin. Figure 3 illustrates the final constructions. Gene transfer was accomplished by co-cultivation (Van den Elzen *et al.*, 1985) between the herbicide-sensitive recipient tobacco protoplasts and the *Agrobacterium tumefaciens* strain LBA4404 (Hoekema *et al.*, 1983) containing the constructions. The cells were divided into two populations and screened in parallel for either antibiotic or herbicide resistance. In each experiment a minimum of 100 kanamycin-resistant transformants were scored. The results of these experiments are collated in Table I. Chlorsulfuron-resistant transformants are listed as a fraction of the number of kanamycin-resistant transformants.

The data in Table I indicate that a class I ALS gene derived from the C3 mutant line conferred resistance to chlorsulfuron, whereas a class II ALS gene derived from the same line did not. Conversely, a class II gene derived from the S4-Hra line conferred chlorsulfuron resistance, while two class I genes derived from this line did not. These data show that the class I ALS gene corresponds to the SuRA locus and is the site of the C3 mutation, while the class II ALS gene corresponds to the SuRB locus and is the site of the S4 and Hra mutations. As shown in Table I, the chlorsulfuron resistance phenotype conferred by the S4-Hra mutant gene was transferred at the same frequency as was the kanamycin resistance phenotype (i.e. the ratio of $Cs^R$ to $Kan^R$ is 1.0). In contrast, the chlorsulfuron resistance conferred by the C3 mutant gene was transferred at only 20–25% of the frequency of the linked kanamycin resistance phenotype. The basis for this difference in the frequency of resistance conferred by the transfer of these two distinct mutant genes is unclear. We suspect it reflects a significant difference in the actual level of resistance to chlorsulfuron conferred by these two genes in the mutant lines. In order to measure these levels, we have compared the growth response of tobacco calli transformed with the mutant ALS

**Table I.** Detection of mutant ALS genes by plant cell transformation

| ALS mutant | S4-Hra | | S4-Hra | | C3 | | S4-Hra | | C3 | |
|---|---|---|---|---|---|---|---|---|---|---|
| ALS gene class | I | | I | | I | | II | | II | |
| Construct no.[a] | pALS011BV | pALS012BV | pALS181BV | pALS182BV | pALS351BV | pALS381BV | pALS032BV | pALS033BV | pALS312BV | pALS313BV |
| $cs^R/kan^R$ [b] | 0 | 0 | 0 | 0 | 0.23 | 0.21 | 1.00 | 0.91 | 0 | 0 |

[a]See Figure 3.
[b]The ratio of transformants resistant to chlorsulfuron to transformants resistant to kanamycin.

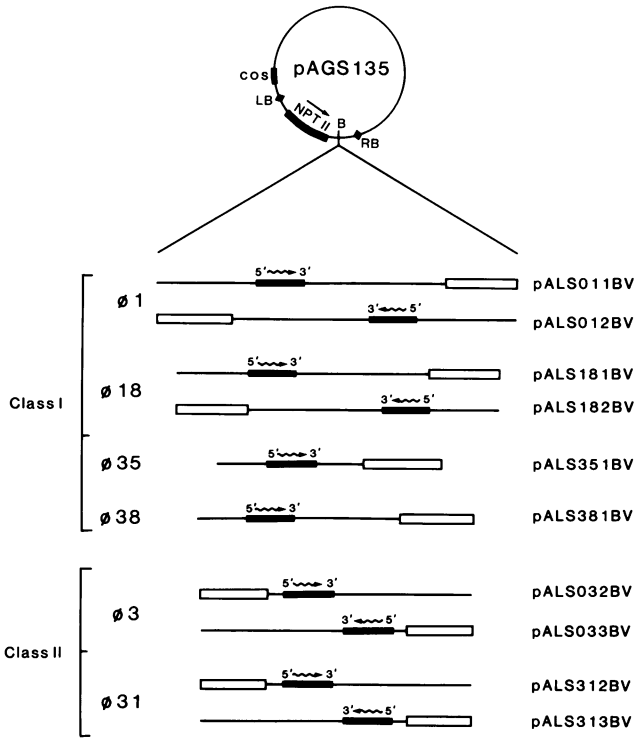



**Fig. 3.** Binary T-DNA vector constructions for introduction of the ALS genes into *N.tabacum*. pAGS135, a binary vector for plant cell transformations, is shown, along with ALS gene inserts in the vector. Restriction fragments carrying ALS genes with their flanking regions were subcloned into pUC vectors. The subclones were then inserted into the *Bam*HI site of pAGS135, so that the ALS and pUC sequences were inserted between the T-DNA borders of the vector. The orientations of the ALS genes relative to the T-DNA borders are shown. The open boxes on the pALS maps represent pUC vector sequences. The solid boxes show the positions of the ALS genes in the pUC plasmids, with the direction of transcription of the ALS genes indicated by wavy arrows. The pAGS135 vector carries a T-DNA left border (LB), a T-DNA right border (RB), the kanamycin-selectable plant marker neomycin phosphotransferase (NPT II), a *Bam*HI (B) cloning site, and a phage λ cos site (cos) for DNA packaging. During plant cell transformation, sequences carried between the T-DNA border sites are transferred to the plant genome.

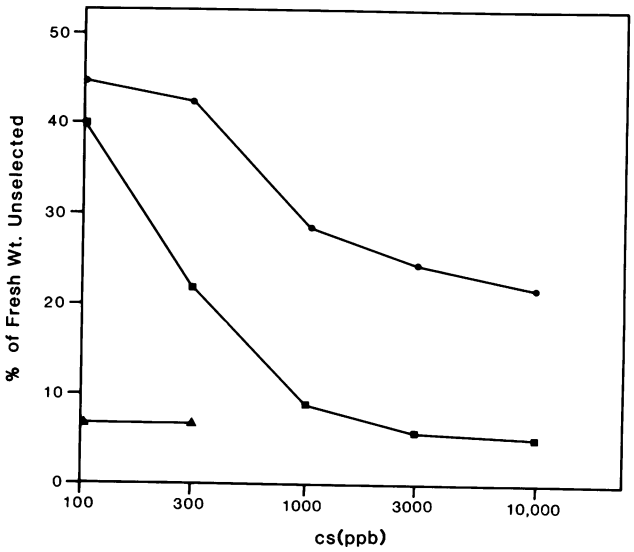



**Fig. 4.** Growth response of transformed tobacco calli. For each measurement, 50 transformed calli were grown for 20 days on the indicated concentrations of chlorsulfuron, and the wet weight of the calli then determined. Unselected calli were grown on medium lacking chlorsulfuron. Growth response in the presence of chlorsulfuron was calculated as a percentage of the fresh weight of unselected calli. The chlorsulfuron concentration is shown in p.p.b. pAGS112 (vector) ▲——▲; pALS381BV(C3) ■——■; pALS032BV(S4-Hra) ●——●.

genes and with the vector pAGS112 (Van den Elzen *et al.*, 1985) to chlorsulfuron. Approximately 50 independent transformants were grown for 20 days at 28°C on increasing concentrations of chlorsulfuron and their wet weight was then measured. The results for each transformed calli population, calculated as a percentage of the unselected growth weight (no chlorsulfuron), are shown in Figure 4. At 100 p.p.b. chlorsulfuron the C3- and S4-Hra-transformed tissues showed similar growth rates. At 300 p.p.b. growth of the C3-transformed tissue was more sensitive to the herbicide than was growth of the S4-Hra-transformed tissue, while at 1000 p.p.b. growth of the C3-transformed tissue was similar to the pAGS112 (control)-transformed tissue and distinctly reduced from that of the S4-Hra tissue. The growth of S4-Hra-transformed tissue declined at chlorsulfuron levels of 1000 p.p.b., but never fell to the control levels even at 10 000 p.p.b. These results are consistent with the levels of herbicide resistance obtained in the original C3 and S4-Hra mutant plant lines and in calli derived from these lines. The level of herbicide resistance expressed in the S4-Hra line was higher than that in either the C3 or S4 lines (Chaleff and Ray, 1984; Chaleff *et al.*, 1986). The different levels of chlorsulfuron resistance observed in the original mutant plant lines and in the transformed tissue could be due to the particular mutations in the two genes, or it could reflect differences in the efficiency of expression of the two classes of ALS genes. We are currently testing these hypotheses.

### *Molecular basis of the S4-Hra and C3 mutation*

Figure 5 compares the nucleotide and deduced amino acid sequences of the SuRA wild-type ALS gene (Mazur *et al.*, 1987) with that of the wild-type SuRB gene. The ALS enzyme is specified by a nuclear gene (Chaleff and Ray, 1984; Mazur *et al.*, 1987) but it is localized within the chloroplast (Miflin, 1974; A.V.Jones *et al.*, 1985). Analogous nuclear-encoded, chloroplast-localized plant proteins which have been analyzed have been shown to have an N-terminal transit peptide which appears to function in transport of the protein into the chloroplast (Schmidt *et al.*, 1979). Based upon comparisons between a tobacco and an Arabidopsis ALS gene, Mazur *et al.* (1987) postulated that the chloroplast transit

**Table II.** ALS reintroduction: level of chlorsulfuron resistance in cells of *N.tabacum* cv. W38 transformed with the S4-Hra mutant ALS gene (number of colonies actively growing after one month on selective media[a])

| | Chlorsulfuron (ng/ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 20 | 50 | 200 | 500 | 2000 | 20 000 | 50 000 |
| *N.tabacum*[b] | 100 | 0 | 0 | 0 | 0 | 0 | ND[d] | ND[d] |
| *N.tabacum*[c] | 100 | 100 | 100 | 100 | 100 | 99 | 6 | 0 |

[a]One hundred colonies plated at each chlorsulfuron level.
[b]Colonies derived from non-co-cultured plant cells.
[c]Colonies derived from co-cultivation with *A.tumefaciens* harboring pALS032BV and selected for chlorsulfuron resistance at 2 p.p.b.
[d]Not done.

peptide for ALS comprises ~85 amino acids at the N terminus of the protein. The nucleotide sequences (1–255) which specify these predicted transit peptide show 23 nucleotide substitutions and three regions of 6–9 base pair duplications/deletions when the SuRA and SuRB genes are compared. The 6-bp sequence at position 10–15 in both genes is repeated in the SuRB gene at position 16–21. Similarly, a 9-bp sequence found at position 26–34 in both genes is repeated in the SuRA gene at position 35–43. At position 157–174, the SuRA gene contains three tandem repeats of a 6-bp sequence, while it occurs only once in the sequence of the SuRB gene. The 23 nucleotide substitutions within the proposed transit peptide coding region result in seven amino acid changes, while the three duplications/deletions result in an additional seven amino acid changes. Thus high divergence in the transit peptide is introduced through in-frame duplications/deletions. Considerable divergence in the transit peptides of individual members of multigene families has been found previously (Dunsmuir, 1985; Dean *et al.*, 1987).

The remainder of the coding sequences in the two genes is highly conserved, showing only 49 changes in the 1752 nucleotides, or 2.7% divergence. Forty-five of these changes are silent; hence there is only a predicted amino acid divergence of 0.7% between the two peptides. These data indicate that the two ALS genes specify closely related proteins. There is significant homology proximal to the coding region, up to position −190. This region includes the TATA box and CAAT box as defined by Mazur *et al.* (1987). There is a limited region of sequence (52 nucleotides) distal to the coding region available for comparison between the two ALS genes; throughout this region the nucleotide sequences are conserved.

The C3 and S4-Hra mutant genes conferring herbicide resistance were sequenced in parallel to their wild-type alleles. Given our finding that there are only two classes of ALS genes in tobacco, we expected that both the S4 and the Hra mutations would occur in the SuRB gene. The sequence of the SuRB gene from the S4-Hra line confirmed this expectation, and also revealed a third, silent mutation in the S4-Hra gene (Figure 6). The S4-Hra gene has a C to G transition at nucleotide 587, which results in substitution of alanine for proline at amino acid 196. Immediately proximal to this mutation, at nucleotide 586, is a third position, a silent G to T mutation. The third substitution in the S4-Hra gene is a G to T change at nucleotide 1719, which results in the substitution of tryptophan for leucine at position 573. The mutant ALS gene from the C3 line has a single nucleotide change relative to its wild-type allele; a C to A transition at nucleotide 588 results in the substitution of a glutamine for a proline at position 196 in the enzyme. The glutamine substitution in the C3 mutant is at the same proline residue as the alanine substitution in the S4-Hra mutant. There are no additional changes in the proximal or distal non-coding regions of either mutant gene.

**Table III.** Sulfonylurea resistance of tobacco plants transformed with pALS032BV [shoot height (% of control)[b]]

| Plant (set number)[a] | Chlorsulfuron (p.p.m.) | | |
|---|---|---|---|
| | 30 | 100 | 300 |
| Sensitive | 3 | 1 | 1 |
| Resistant | | | |
| 3 | 44 | 25 | 19 |
| 5 | 75[c] | 65[c] | 15 |
| 6 | 74[c] | 79[c] | 74[c] |
| 7 | 68[c] | 32 | 26 |
| 8 | 33 | 22 | 11 |
| 9 | 80[c] | 70[c] | 75[c] |
| 10 | 89[c] | 37 | 21 |
| 12 | 16 | 11 | 11 |
| 15 | 17 | 28 | 22 |
| 18 | 75 | 88 | 38 |
| 20 | 25 | 31 | 38 |
| 25 | 100[c] | 47 | 26 |
| 29 | 56[c] | 29 | 14 |
| 31 | 63 | 18 | 18 |
| 32 | 23 | 23 | 15 |
| 35 | 30 | 15 | 15 |

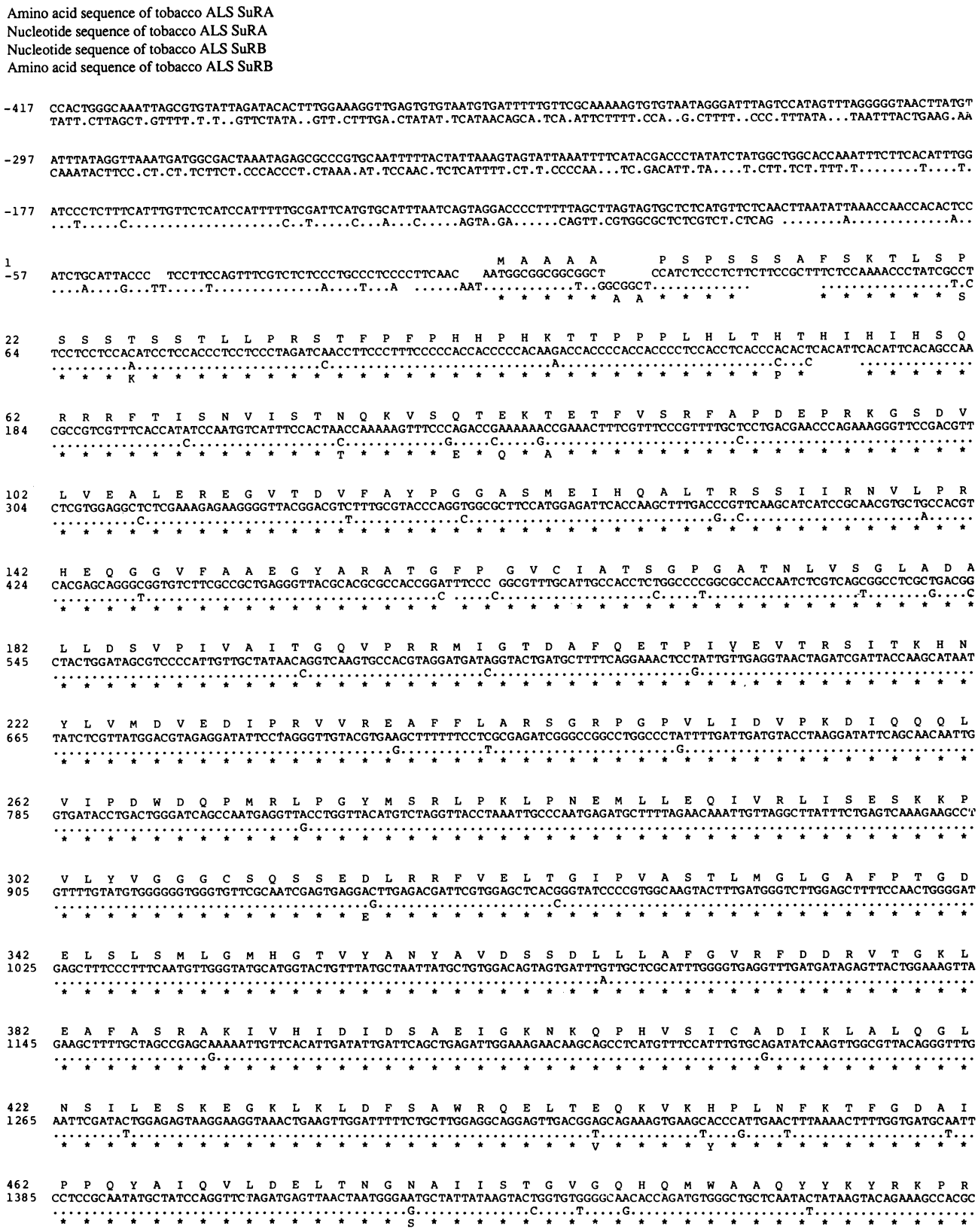
[a]Each set of four plants was regenerated from a single protoplast derived callus. The sensitive plants were regenerated from a callus derived from non-co-cultured cells. The resistant plants, designated by a number, come from calli derived from cells co-cultured with *A.tumefaciens* harboring pALS032BV and selected for resistance to chlorsulfuron at 2 p.p.b.
[b]At the four- to five-leaf stage, sets of four plants were sprayed with 5 ml of an aqueous solution containing the indicated concentration of chlorsulfuron, 0.2% Tween 80 and 10% (by vol) acetone. Controls received 5 ml of a solution lacking chlorsulfuron. Shoot height was measured ~5 weeks after treatment. At this time all plants not sprayed with chlorsulfuron were flowering.
[c]Plant flowering 5 weeks after treatment.

In summary, these sequence data show that a single amino acid change at Pro-196 in the tobacco ALS enzyme specified by the SuRA locus changes the plant phenotype from herbicide-sensitive to herbicide-resistant. Furthermore, two separate substitutions, one at Pro-196 and a second at Trp-573 in the SuRB locus, also result in a herbicide resistance phenotype. The double mutation enhances the herbicide resistance ~10-fold in the original mutant lines (Chaleff *et al.*, 1986), and also enhances resistance in transformed tissue. At present, we do not know which of the two mutations in the S4-Hra mutant gene is the Hra mutation. We do not know whether the effect of the secondary Hra mu-

tation is simply additive with the S4 mutation or if the Hra mutation alone could confer the resistance phenotype at such high levels. There are two examples of molecularly characterized ALS genes in other species (*Escherichia coli* and yeast) which are responsible for a sulfonylurea herbicide resistance phenotype. In each case there is a single amino acid replacement within a conserved region of the two proteins; the yeast ALS gene mutation occurs at the position corresponding to Pro-196 of the plant gene (Falco and Dumas, 1985; Yadav *et al.*, 1986).

### *Transgenic plants derived from transfer of the S4-Hra mutant ALS gene are highly resistant to sulfonylurea herbicides*

Table II shows the degree of resistance to chlorsulfuron of



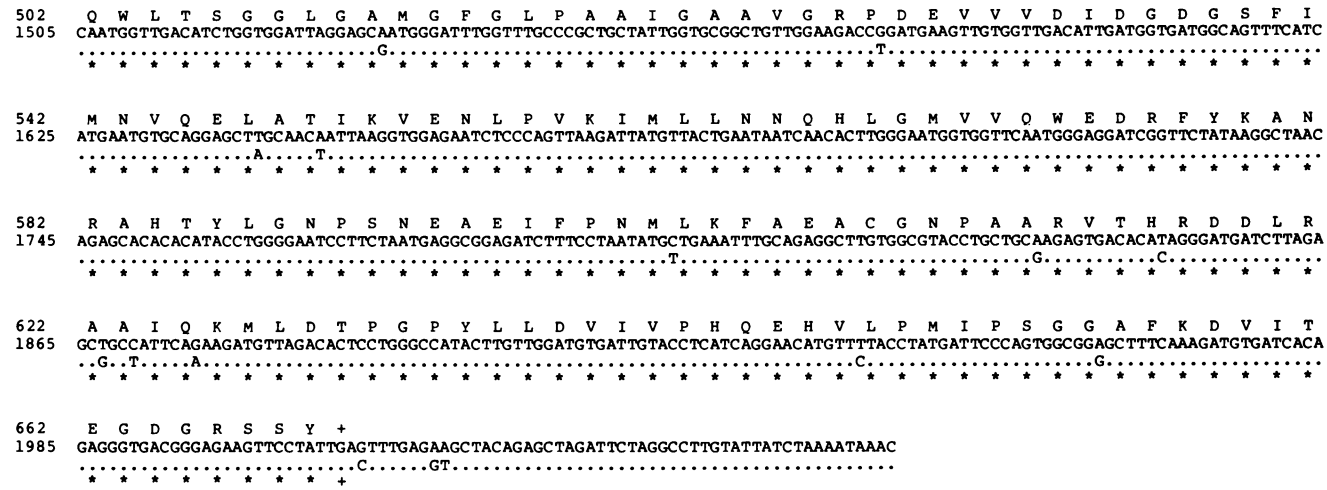



**Fig. 5.** Comparison of the nucleotide and deduced amino acid sequences of SuRA and SuRB wild-type ALS genes. The nucleotide sequence of the SuRA ALS gene is shown on the top line, and the sequence of the SuRB ALS gene is shown on the bottom line. In the coding regions of the genes, the deduced amino acid sequences of the genes are shown above or below their respective nucleotide sequences. Nucleotides that are common to both gene classes are depicted with dots; identical amino acid residues are shown with stars. In order to align the genes, spaces were inserted into the sequences to compensate for deletions relative to the other sequence. Nucleotide 1 is shown as the A of the first coding ATG residue; nucleotides preceding this A are given negative numbers.

*N. tabacum* cells transformed with the S4-Hra mutant ALS gene. Isolated calli between 1 and 2 mm in diameter, which were selected for resistance to chlorsulfuron at 2 ng/ml, were plated at chlorsulfuron concentrations ranging from 0 to 50 μg/ml. The results shown in Table II demonstrate that all transformed calli resistant to 2 ng/ml chlorsulfuron were also resistant to 500 ng/ml chlorsulfuron, that 99% were resistant to 2000 ng/ml chlorsulfuron and that 6% were resistant to 20 000 ng/ml chlorsulfuron. The control cells transformed with the vector grew only on media lacking herbicide.

Plants were regenerated from transformed calli and the resulting transgenic plants were tested for herbicide resistance. At least four plants were regenerated from each of 16 independent transformants, and together with non-transformed control plants were tested in a greenhouse for the effect of foliar applications of herbicide. Since protoplast co-cultivation had been used for the transformation, all four plants derived from each independent transformed callus were assumed to be genetically identical clones. The results of these experiments are summarized in Table III, in which the herbicide resistance of the transgenic plants was measured by comparing the averaged shoot heights of treated plants with those of non-treated sibling controls. At least one-third of the transformants were not markedly affected by a herbicide application of 30 p.p.m., in so far as they attained a shoot height, after spraying, which was at least 75% of the unsprayed sibling plants. In contrast, the untransformed control plants, after herbicide application, attained a shoot height which was only 3% of that for their unsprayed sibling controls. At 100-fold higher herbicide concentrations two of the 15 transformants attained a shoot height which was 75% that of the unsprayed control. The most probable explanation for the variable levels of resistance shown by the independently transformed clones is that there are different levels of expression of the transferred mutant gene in the clones. Clone to clone variation in the expression of introduced genes in transgenic plants is a phenomenon which has been observed (J.D.G.Jones *et al.*, 1985).

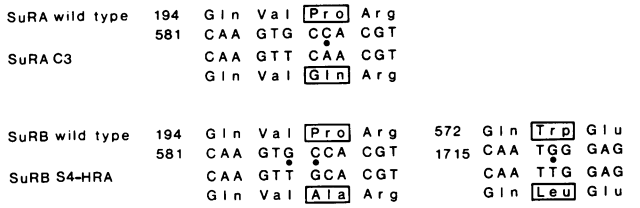



**Fig. 6.** The molecular basis of the S4-Hra and C3 mutation. The nucleotide and deduced amino acid sequences surrounding the S4-Hra and C3 mutations are shown. Nucleotide substitutions are indicated with dots, and amino acid substitutions are indicated with boxes. Numbers are taken from Figure 5.

### *Conclusion*

We have demonstrated that sulfonylurea herbicide resistance in tobacco can result from a single amino acid substitution in the mature peptide of ALS. In tetraploid *N. tabacum* there are two distinct ALS genes. A single amino acid change in one of these ALS genes resulted in the production of an enzyme which was resistant to the herbicide. Two amino acid changes in the other ALS gene produced a protein which was even more resistant to herbicide. Introduction of these mutant genes into sensitive tobacco cells produced transgenic tobacco plants which were highly resistant to herbicide. These data suggest that it will be possible to introduce sulfonylurea resistance into crops by isolating the ALS gene from a species, mutagenizing the gene at specific sites and transferring the mutant gene to sensitive plants. Alternatively, introducing one of the resistant tobacco ALS genes described here into different species may also produce a herbicide-resistant phenotype in the recipient plant.

## Materials and methods

### *Genomic filter hybridization*

Plant genomic DNA was prepared from tobacco leaves as previously described (Dunsmuir *et al.*, 1983). Genomic DNA (10 μg) was digested with *Eco*RI or *Nco*I and separated by electrophoresis through a 0.8% agarose gel. The DNA was transferred to nitrocellulose by the Southern blot procedure (Southern, 1975) and hybridized to $^{32}$P-labeled RNA riboprobes in 50% formamide, 10% dextran sulfate, 10 × Denhardt's solution, 100 μg/ml salmon sperm DNA, 25 mM $Na_2HPO_4$, pH 7, 25 mM $NaH_2PO_4$, pH 7,

0.6 M NaCl, 0.1% SDS for 2 days at 42°C. The filters were washed in 2 × SSC, 0.1% SDS at room temperature and then in 0.1 × SSC, 0.1% SDS at 65°C for 60 min. Probes were prepared by subcloning restriction fragments containing 5′ or 3′ ALS sequences (see Figure 2) into SP6 vectors (Melton *et al.*, 1984), and carrying out the SP6 polymerase reactions according to the protocol obtained from the manufacturer (Promega, Madison, WI).

***Construction and screening of genomic libraries***

Plant DNA was partially digested with *Sau*3A, size-fractionated on a 10–40% sucrose density gradient, inserted into the *Bam*HI site of EMBL3 λ phage (Frischauf *et al.*, 1983) and packaged *in vitro* (Hohn and Murray, 1977). 600 000 recombinant phage from each library were screened by hybridization to the 5′ tobacco ALS gene probe as described above, except that dextran sulfate was omitted. Filters were washed at 42°C. Positive phage were purified, screened with a 3′ ALS gene probe, and the phage DNA isolated as described in Dean *et al.* (1985).

***Construction of binary vectors for ALS gene reintroduction into N.tabacum***

Restriction fragments containing the ALS gene and the adjacent 5′ and 3′ regions were subcloned into pUC vectors (Norrander *et al.*, 1985). The subclones were linearized by complete or partial *Bam*HI digestion and ligated into the *Bam*HI site of pAGS135. pAGS135 is equivalent to the binary vector pAGS112 (Van den Elzen *et al.*, 1985), except for the deletion of the *Xho*I site adjacent to the right border. The ligated DNA was used to transform *E.coli* strain HB101, or was packaged into phage heads *in vitro* and used to transfect strain HB101. The orientation of the ALS gene relative to the T-DNA borders in the binary vector was determined by restriction enzyme analysis. The binary vectors were transferred by triparental mating with *E.coli* strain HB101/pRK2013 (Figurski and Helinski, 1979) into *A.tumefaciens* strain LBA4404 (Hoekema *et al.*, 1983), which contains the vir region of the Ti plasmid. Transconjugants were selected on LB medium (Miller, 1972) containing rifampicin (100 μg/ml) and tetracycline (1 μg/ml). Plasmids were maintained in strain LBA4404 on Minimal A medium (Miller, 1972) containing tetracycline (1 μg/ml) and streptomycin (500 μg/ml).

***DNA sequence analysis***

Sequencing was carried out by the dideoxy chain termination procedure (Sanger *et al.*, 1977). Both strands of each ALS gene were sequenced by generating *Bal*31 exonuclease deletions as described in Dean *et al.* (1985), or by using synthetic oligonucleotide primers.

***Transfer of ALS genes to tobacco cells***

*N.tabacum* cv. Wisconsin 38 was maintained as an axenic culture on the agar solidified medium of Murashige and Skoog (1962). Cultures were maintained in 3 × 4-in. Magenta® boxes at 28°C with 'cool white' fluorescent light at 6000 lux with a 16-h light/8-h dark photoperiod. Co-cultivation of protoplast-derived tobacco cells with *A.tumefaciens* was carried out as described previously (Van den Elzen *et al.*, 1985). Selection for kanamycin and chlorsulfuron resistance was initiated 8 days after protoplast isolation. Co-cultured plant cells were collected by centrifugation at 50–70 *g* for 5 min and the cultured media was replaced with fresh media containing either kanamycin (50 μg/ml) or chlorsulfuron (2 ng/ml). When scoring the frequency of transfer of herbicide resistance compared with kanamycin resistance, cultures were divided and then selected in parallel on the appropriate media. Media were replaced twice weekly. Resistant colonies were immobilized in agarose ~20 days after protoplast isolation and counted 7–10 days later. Plants were regenerated by placing colonies on the media of Murashige and Skoog containing 0.1 μg/ml NAA, 1.0 μg/ml BAP and 10 ng/ml chlorsulfuron. Multiple shoots were derived from single colonies and these were moved in sets onto hormone-free medium (MS) to allow root formation. Rooted plantlets were moved into small peat pots containing a 1:1 mixture of Perlite and supersoil and grown for 1 week in an environmental chamber at 80% relative humidity and then transferred to a greenhouse.

***Growth response of transformed tobacco calli***

Calli were produced by co-cultivation of protoplasts of *N.tabacum* cv. Wisconsin 38 with *Agrobacteria* containing the binary vector pAGS112 (control), pALS381BV (C3 mutant ALS gene) or pALS032BV (S4-Hra mutant gene). Intial selection for transformation was with kanamycin at 50 μg/ml 8 days after protoplast isolation. At 30 days after isolation 50 individual resistant calli from each co-cultivation were placed on Murashige and Skoog agar solidified media containing NAA at 1 μg/ml and BAP at 0.1 μg/ml, with or without chlorsulfuron. These calli were cultured for 20 days at 27°C with a 16-h light/8-h dark photoperiod. Growth was calculated for each concentration as a percentage of fresh weight of unselected calli.

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