Regulation of Tobacco Acetolactate Synthase Gene Expression

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Acetolactate synthase (ALS) catalyzes the first common step in the biosynthesis of isoleucine, leucine, and valine. The previous cloning of two tobacco (Nicotiana tabacum) ALS genes (SurA and SurB) has allowed transcript accumulation from these genes to be monitored. mRNA blot analysis of ALS transcripts showed a message size of 2.2 kb. Quantitation of the levels of ALS messages in tobacco organs indicated that there was a 3- to 4-fold variation in transcription of the two tobacco ALS genes examined, and the expression levels of the two tobacco ALS genes were then followed separately. Both tobacco ALS genes are expressed in a coordinated manner in all tobacco organs examined, with the SurB gene being consistently expressed at higher levels than the SurA gene.

ALS (EC 4.1.3.18) catalyzes the first common step in the biosynthesis of the essential amino acids Ile, Leu, and Val in bacteria, yeast, and higher plants. Bacteria have multiple isozymes of ALS, whereas yeast has a single ALS protein found in mitochondria (Ryan and Kolhaw, 1974). Higher plants have one or more ALS isozyme localized in the chloroplasts (Miflin, 1974; Jones et al., 1985). The regulation of ALS activity in plants is of interest, partly due to the finding that several classes of herbicides, including sulfonylureas, imidazolinones, and triazolopyrimidines, inhibit the activity of this enzyme (Mazur and Falco, 1989). Inhibition of ALS activity in bacteria by these compounds leads to amino acid starvation and to the accumulation of toxic precursors (LaRossa et al., 1987). In plants, the toxicity of these herbicides can be reversed by supplementation with branched-chain amino acids (Ray, 1984).

In tobacco (Nicotiana tabacum), mutant lines resistant to sulfonylureas were isolated by selection in tissue culture. Genetic analyses of these mutant lines defined two unlinked loci that were designated SurA and SurB (Chaleff and Ray, 1984). The herbicide-resistant phenotypes of these isolates were shown to be due to altered ALS proteins (Chaleff and Mauvais, 1984). Using an ALS gene isolated from yeast as a heterologous hybridization probe, ALS genes were isolated from Arabidopsis thaliana and N. tabacum, and their DNA sequences were determined (Mazur et al., 1987). N. tabacum, an allotetraploid, is thought to have arisen from the hybridization of the diploid species Nicotiana tomentosiformis and Nicotiana sylvestris (Goodspeed, 1954; Gray et al., 1974). By combining genetic analyses with DNA blot hybridizations, it was determined that the SurB gene derived from the progenitor N. tomentosiformis, and that the SurA gene derived from the ancestral N. sylvestris (Lee et al., 1988). Sequence analyses of the two ALS genes from N. tabacum showed that they share about 95% nucleotide sequence identity in the protein coding regions (Lee et al., 1988). In the 5' noncoding region of these genes, greater than 80% identity extends to about 170 bases upstream from the ATG translational start site.

That both ALS genes of N. tabacum are functionally expressed can be deduced from the observation that mutations in the protein coding regions of either gene are able to confer herbicide resistance in plants (Chaleff and Bascomb, 1987). Moreover, cloned gene segments containing an herbicide-resistant form of either gene can be used to transform sensitive lines of tobacco to herbicide resistance (Lee et al., 1988). DNA blot analyses have indicated the presence of at least two ALS genes in both soybean and corn species (S.J. Keeler, unpublished results), whereas some Brassica species have multiple ALS genes (Wiersma et al., 1989). However, multiple ALS genes do not seem to be essential to the normal growth of higher plants because both A. thaliana and sugarbeet have only one ALS gene (Mazur et al., 1987; B.J. Mazur, unpublished data).

Maintenance of more than one functional ALS gene may result from a need for variable expression of these genes in different organs or during different developmental stages. Alternatively, different ALS isozymes may be produced in response to particular environmental conditions or changes in cellular growth rates, as has been suggested for the multiple ALS enzymes of Escherichia coli (Umbarger, 1983). However, the presence of multiple copies of ALS in tetratetraploid tobacco may simply reflect the evolutionary origin of this species. In this paper, we examine the organ and tissue specificity of expression of ALS genes from the tetratetraploid N. tabacum and

Abbreviations: ALS, acetolactate synthase; EPSPS, 5-enolpyruvyl-shikimate-3-phosphate synthase.
determine the transcriptional start sites and relative expression levels of each ALS gene in different organs.

MATERIALS AND METHODS

Plasmids

The five plasmids used to study ALS gene expression are described in Table I. ALS gene segments were cloned into the vectors pSP64 (Promega) (pTS clones) or pTZ18/19 (Pharmacia) (pTZ and pS clones) using standard cloning procedures as described by Maniatis et al. (1982). Transcripts were produced from these plasmids in vitro using either T7 or SP6 polymerase, following linearization of plasmids with the enzymes listed in Table I.

Plant Material

Tobacco plants used for RNA extractions were Nicotiana tabacum cv. Xanthi grown from seeds in 6-inch pots of soil until they became 3 to 4 inches tall (26 d). They were then transplanted into individual 6-inch pots of soil or sand and watered with Hoagland nutrient solution (Hoagland and Arnon, 1938). Plants were maintained in growth chambers at 24°C day/20°C night with a 12-h photoperiod. Leaves and stem sections were harvested from separate plants grown in sand at 42 d after seeding. A few plants were allowed to grow to maturity until they became 3 to 4 inches tall (26 d). They were then transplanted into individual 6-inch pots of soil or sand and their fully opened flowers were harvested. Roots were harvested from separate plants grown in sand at 42 d after seeding. Material was frozen in liquid nitrogen and used immediately or it was stored at -70°C.

Plants used for in situ experiments were N. tabacum cv Samsun grown to maturity under greenhouse conditions. Leaf samples were taken from 5-cm leaves, and stem sections were from plants varying in size from 4 to 7.5 cm. Root tips were collected from hydroponically grown plants approximately 30 cm in height.

RNA Extractions and Hybridizations

Total RNA was prepared by grinding approximately 4 g of frozen plant material in liquid nitrogen, digesting the fine powder with 10 mg of proteinase K in 20 mL of extraction buffer (50 mM Tris, pH 9.0, 10 mM EDTA, 2% SDS) at 50°C, and carrying out two phenol:chloroform extractions and one chloroform extraction followed by ethanol precipitation. The pellet was dried and resuspended in 5 mL of diethyl pyrocarbomate-treated water and extracted twice with 2 M LiCl2 (Hall et al., 1978). The RNA pellet was dissolved in 5 mL of sterile diethyl pyrocarbonate-treated water, and aliquots were stored frozen at -20°C.

For mRNA blot analyses, 10- to 20-μg aliquots of each total RNA sample were denatured by heating at 65°C in 32% formamide, 3.5% formaldehyde in Tris-borate buffer for 20 min, and the samples were then loaded onto 1.0% agarose gels containing 3.0% formaldehyde in Tris-borate buffer (Rave et al., 1979) along with RNA size standards (BRL). Gels were subjected to electrophoresis overnight at 40 V, and then stained with ethidium bromide in 50 mM NaOH for 0.5 h and destained in 1 M sodium acetate, pH 5.2, for 1 h. A photograph of the stained gel was used to determine the relative levels of rRNA bands in each sample. The RNA was then blotted onto Zeta Probe nylon transfer membranes (Bio-Rad) with 20× SSC (Maniatis et al., 1982).

Anti-mRNA probes used for detection of the tobacco ALS transcripts on gel blots or for in situ hybridizations were prepared by transcription of BamHI-linearized plasmid pTZALS.1, using T7 polymerase and [α-32P]UTP. The in vitro transcription procedure recommended by the manufacturer (Promega) was used with the addition of 2.5 μL of vanadyl ribosides (BRL).

Hybridizations were carried out in a solution containing 50% formamide, 5× SSC, 3× Denhardt’s solution (Maniatis et al., 1982), 0.5% SDS, 100 μg/mL of sheared calf thymus DNA, and 2.5% dextran sulfate at 55°C for more than 12 h with 1 million TCA-precipitable cpm added per mL of hybridization mix. The filters were washed three times in 2× SSC, 0.1% SDS at room temperature and were then washed three times in 0.1× SSC, 0.1% SDS at 65 to 70°C for a total of 3 h.

Quantitation of ALS Messages in Tobacco Organs

To quantitate ALS message levels in tobacco samples, 2 μg of total RNA per sample were bound to nylon membranes using the procedure of Kaufmann et al. (1984), hybridized with a 32P-labeled anti-mRNA probe, and subjected to liquid scintillation counting. The tobacco SurA anti-mRNA probe hybridized readily to both the SurA and SurB in vitro transcripts. The number of picograms of ALS message per microgram of total RNA in each plant organ sample was calculated by comparison with a standard curve. This standard curve was generated using unlabeled ALS mRNAs synthesized by

<p>| Table I. Plasmids used to generate SurA and SurB ALS gene transcripts |
|--------------------|---------------------|----------------|----------------|----------------|</p>
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Allele</th>
<th>DNA Segment</th>
<th>RNA Strand</th>
<th>RNA Size</th>
<th>RNA Polymerase</th>
<th>Termination Site</th>
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<tr>
<td>pTSNTC</td>
<td>SurB</td>
<td>+348 to -133</td>
<td>anti-mRNA</td>
<td>520 bases</td>
<td>SP6</td>
<td>EcoRI</td>
</tr>
<tr>
<td>pTZNNTD</td>
<td>SurB</td>
<td>-133 to +1767</td>
<td>mRNA</td>
<td>1900 bases</td>
<td>T7</td>
<td>SalI</td>
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<tr>
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<td>anti-mRNA</td>
<td>588 bases</td>
<td>SP6</td>
<td>EcoRI</td>
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<tr>
<td>pSAXB</td>
<td>SurA</td>
<td>-177 to +1776</td>
<td>mRNA</td>
<td>1955 bases</td>
<td>T7</td>
<td>BglII</td>
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<tr>
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The sizes of the protected fragments were determined by 6% RNase A were separated on denaturing acrylamide gels. Fragments remaining after digestion with RNase T1 and termini, or to 10 μg of total RNA from plant material. Labeled annealed to control in vitro transcripts with known 5' mRNA of Melton et al. (1984). Labeled anti-mRNA probes were function in the range of 5 to 200 pg of message. Counts versus picograms of ALS message; this was a linear ards was used to generate reference curves of hybridized Hybridization of labeled anti-mRNA probes to these stand- bound to filters after dilution with 2 μg of tRNA carrier. (SurA) (SurB). pTZNTD and pSAXB The resulting samples large-scale in vitro transcription reactions of the templates pTZNTD (SurA) and pSAXB (SurB). The resulting samples were quantitated spectrophotometrically, and aliquots were bound to filters after dilution with 2 μg of tRNA carrier. Hybridization of labeled anti-mRNA probes to these standards was used to generate reference curves of hybridized counts versus picograms of ALS message; this was a linear function in the range of 5 to 200 pg of message.

RNase Protection Assays

RNase protection assays were carried out using the protocol of Melton et al. (1984). Labeled anti-mRNA probes were annealed to control in vitro transcripts with known 5' mRNA termini, or to 10 μg of total RNA from plant material. Labeled fragments remaining after digestion with RNase T1 and RNase A were separated on 6% denaturing acrylamide gels. The sizes of the protected fragments were determined by

In Situ Hybridizations

In situ hybridizations were carried out with sections of paraffin-embedded tobacco organs from N. tabacum cv Sam-sun, as described by Cox and Goldberg (1988). 35S-Labeled anti-ALS mRNA and 35S-labeled ALS mRNA probes were synthesized from pTZALS.1 and pSAXB, respectively, and hybridized to sections. Positive hybridization control probes included 35S-labeled anti-rRNA synthesized from radish 25S and 18S rRNA DNA sequences (Delsany et al., 1983) and 3Hpoly(U). Negative hybridization control probes were 35S-labeled mRNA synthesized from radish rRNA DNA sequences and ALS sense-strand mRNA. Only the results of in situ hybridizations with anti-ALS mRNA and sense-rRNA mRNA probes are shown in Figures 2, 3, and 4. Photographs were processed for both bright-field (0.05% toluidine blue stained) and dark-field microscopy, using Kodacolor Gold 100 film.

RESULTS

Representation of ALS mRNAs in Tobacco Organs

To determine the size and accumulation patterns of ALS mRNAs in tobacco organs, RNA gel-blot analyses were performed. Tobacco plants were dissected as depicted in Figure 1A and RNA was extracted from the indicated organs. RNA gel-blot analyses were done using a SurA anti-mRNA transcript as a hybridization probe and showed that the ALS message is 2.2 kb in length, as illustrated in Figure 1B. This size had been anticipated from the nucleotide sequence of the tobacco ALS gene, which indicated that the SurA gene contained 2.0 kb of coding nucleotides with no apparent introns (Mazur et al., 1987). Figure 1B shows the variation in expression levels of the tobacco ALS messages in different organs. The highest levels of expression were found in seedlings and young leaves, whereas intermediate levels were found in nodal and internodal stem sections and in flowers, and the lowest levels were found in mature leaf samples. Hybridization to RNA extracted from 66-d-old roots showed virtually no ALS message present (data not shown), whereas RNA extracted from 42-d-old roots (Fig. 1B) showed intermediate levels of ALS message. There was some degradation of ALS message in the latter root sample, as evidenced by the lower molecular mass bands seen in lane 8 of Figure 1B. The higher molecular mass bands present in lanes 5 and 6 were not observed in other northern analyses. Within the flower, accumulation was high in anthers, pistils, and petals (data not shown). ALS message has also been observed in RNA extracted from tobacco seeds, but the levels have not been quantitated relative to those found in other organs (J.A. Rafalski, personal communication).

To quantitate the ALS mRNA present in different organs, message levels were assayed by hybridization to labeled anti-ALS probes using the levels of ALS message comparisons with RNA and DNA size standards. The 5' termini of the endogenous ALS transcripts were determined from the lengths of the labeled fragments remaining after RNase digestion.
Figure 2. In situ localizations of ALS gene expression in tobacco vegetative organs. Organs were fixed, embedded in paraffin, sectioned at 10-μm intervals, hybridized with 35S-labeled anti-ALS mRNA and 35S-labeled sense-rRNA mRNA (control) probes, and subjected to autoradiography. Grains were counted in three to five separate grids over tissue sections and on the same slide background counts were taken where there were no tissue sections. Means and SE values for grain counts are included in the table below. A, D, G, Bright-field photographs. A, Root (X25): RC, root cap; M, meristem; C,
present per μg of total RNA in these organs. The amount of combined ALS messages varied between organ types, from a maximum of 10.5 pg/μg of RNA in young leaves to a minimum of 2.5 pg of ALS transcript/μg of RNA in mature leaves. These levels correspond to approximately 0.1% of total mRNA.

In Situ Localization of ALS Gene Transcripts

To determine whether there was tissue specificity in ALS gene expression, a 35S-labeled SurA anti-mRNA probe was hybridized to sections of tobacco vegetative and floral organs. Hybridization was seen in most cell types of the organs studied, as shown in Figures 2, 3, and 4.

Hybridization in the root showed a gradient of gene expression (Fig. 2B). Intense hybridization signals were seen in the most rapidly growing regions, with a decrease in the signal intensities in older cells farther away from the root tip. This gradient in gene expression is consistent with the less-intense hybridization signal to root message seen in the RNA gel-blot analyses; because an entire root system was used as a source of mRNA for those experiments, the ALS message concentrated in the root tips would have been diluted by the mass of the root system.

Grains were counted in five separate grids over leaf and background sections on the same in situ hybridization slide. Leaf sections showed low levels of hybridization signals, three to four times over background (Fig. 2E). Hybridization to internodal stem sections was about two times over background except in the vascular cells, where the intensity of the signal was four times over background (Fig. 2H). This intensity of the signal seen in vascular cells of stem sections was particularly evident in developing nodes (not shown). The localization of hybridization signals in the vascular regions of stem sections paralleled that seen with poly(U) and anti-rRNA probes, which were used as positive hybridization controls. This may indicate an increase in mRNA production in these cells or it may be due to a smaller cell size in the vascular region.

Figures 3 and 4 are examples of the in situ hybridization results seen in floral organs. Anther stage 1 (Koltunow et al., 1990) sections had low levels of hybridization signal with increased signal in tapetal cells, indicated by an arrow in Figure 3B. Stage 12 flower petals had intermediate hybridization signals throughout the organ (Fig. 3D). A developmental series of pistil sections displayed a relatively constant level of hybridization throughout development, but with an increased level of signal in the stigma over the style (stage 8, Fig. 3F). The clearest tissue-specific expression of ALS genes was evident in the ovary, late in pistil development (stages 10 and 12), where hybridization was seen over some embryo sacs and placental tissue, as indicated in Figure 4B (stage 12). This hybridization pattern was not observed when anti-rRNA or poly(U)* was used as a positive control probe (data not shown).

The ALS [35S]mRNA control probe also showed hybridization under the conditions used for the in situ experiments, but tissue localization generally followed a different pattern from that detected by the anti-ALS mRNA probe. When assayed on an RNA gel blot, this sense-strand mRNA probe hybridized to an approximately 1-kb poly(A)* message (data not shown), but was eluted following a 5°C increase in wash temperature. In contrast, hybridization of the anti-ALS mRNA probe was therefore presumed to hybridize to an unrelated mRNA species under the conditions used for the in situ analyses. Because of this spurious hybridization, sense-strand rRNA probes were used as negative controls for in situ hybridizations.

Only background levels of hybridization were seen when using a sense-strand rRNA probe in the in situ experiments (Fig. 2C, F, I; Fig. 3G; Fig. 4C). Anti-rRNA and poly(U) probes were used as positive control probes, and they hybridized well to all tissue sections (data not shown).

Relative Accumulation of the SurA and SurB Transcripts in Tobacco Organs

To compare the relative accumulation of the SurA and SurB transcripts in organs, RNase protection experiments were performed that allowed the two transcripts to be distinguished. The 5' noncoding regions of the tobacco SurA and SurB genes are more than 80% homologous. Using the clones described in Table I, anti-mRNA probes specific for the 5' region of each gene were synthesized. When a 588-base anti-mRNA probe to the SurA gene made using pTSNTC was annealed to total RNA from tobacco seedlings, subsequent RNase digestion resulted in products of about 417 and 200 bases (Fig. 5A, lane 1). The 417-base band ran as a triplet on higher resolution gels. Similar experiments using a 520-base anti-mRNA probe to the SurB gene made using pTSNTC resulted in a primary RNase digestion product of approximately 409 bases, along with smaller fragments (Fig. 5B, lane 1). The smaller products from each reaction are presumed to

(Continued from facing page.) cortex; Pc, procambium; Pd, protoderm; D, Leaf (x66): E, epidermis; P, palisade parenchyma; S, spongy parenchyma; V, vascular bundle. G, Stem (x31.25): E, epidermis; C, cortex; EP, external phloem; X, xylem; IP, internal phloem; Pj, pith. B, E, H. In situ hybridization of an anti-ALS mRNA probe with the respective sections seen in A, D, and G. White grains represent RNA/RNA hybridization. D, Grain counts over leaf sections were mean = 1118 and se = 130.12, and for background they were mean = 289 and se = 14.87. G, Grains were counted in separate grids over pith and vascular regions. Results for grain counts over the pith were mean = 430 and se = 42.56, for the vascular region they were mean = 1163 and se = 35.92, and for background they were mean = 231 and se = 13.64. Photographs were taken with dark-field microscopy and slides were exposed for 12 d. C, F, I, In situ hybridizations of sense-rRNA mRNA probe with root (x50), leaf (x66), and stem (x25) sections, respectively. White grains represent background hybridization levels. Photographs were taken with dark-field microscopy and all slides were exposed for 3 d.
Figure 3. In situ localizations of ALS gene expression in tobacco floral organs. Organs were collected, fixed, embedded in paraffin, sectioned to a 10-μm thickness, hybridized with either 35S-labeled anti-ALS mRNA or 35S-labeled sense-rRNA mRNA probes, and subjected to autoradiography. A, C, E, Bright-field photographs. A, Anther stage 1 (×25): E, epidermis; En, endothecium; Tp, tapetum; V, vascular; Tds, tetrads; C, connective; St, stomium. C, Petal (×13.2): E, epidermis; P, parenchyma; V, vascular bundle. E, Pistil stage 8 (×13.2): St, stigma; Sy, style; TTI, transmitting tissue. B, D, F, In situ hybridization of anti-ALS mRNA probe with the respective sections seen in A, C, and E. White grains represent RNA/RNA hybridization. Photographs were taken with dark-field microscopy and slide exposure times were: B, 12 d; D, 9 d; and F, 10 d. G, In situ hybridization of sense-rRNA mRNA probe with pistil section, stage 8 (×13.2). White grains represent background hybridization levels. Photographs were taken with dark-field microscopy and slide exposure time was 6 d.

result from the annealing of the probes to message from the opposite (nonidentical) ALS gene (Fig. 5A, lane 5; Fig. 5B, lane 4). Control experiments using in vitro-generated sense-strand transcripts of each gene indicated that full-length RNase digestion products were obtained only when the probes were annealed to their homologs, as seen in Figure 5A, lane 4, and Figure 5B, lane 5. As a control for nonspecific annealing, both probes were annealed to an in vitro-generated Arabidopsis ALS gene mRNA and digested with RNase; no probe fragments large enough to be retained on the gel remained (Fig. 5A, lane 3; Fig. 5B, lane 3).

These experiments allowed us to calculate the positions of the 5' ends of each tobacco ALS gene transcript. The SurA gene transcription initiation site is at —54, —58, and —63 bp, while the SurB gene transcription initiation site is at —60 bp. These sites are depicted with arrows in Figure 6.

RNA was then extracted from leaves, stems, roots, and flowers and subjected to similar RNase protection analyses; the results are shown in Figure 7. The 409-base digestion product was produced from the SurB gene probe (Fig. 7, lanes 2, 4, 6, and 8), whereas the three digestion products around 417 bases were produced using the SurA gene probe (Fig. 7, lanes 1, 3, 5, and 7). The identical sizes of the labeled digestion products in all four organ samples indicate that the transcripts of both genes were initiated at the same sites in each organ. Further, the equivalence in the relative intensities of these hybridization products indicates that the relative accumulation of the two genes is constant in each organ.
Because equivalent amounts of probe were added to each reaction, the greater hybridization of each probe to the \textit{SurB} message, as reflected in the breakdown products in Figure 5A, lane 1, and in the full-length products in Figure 5B, lane 1, and in the stronger signal in all \textit{SurB} lanes in Figure 7, indicates that the \textit{SurB} message is more abundant than the \textit{SurA} message. Similar results were found with RNA derived from seedlings or from 66-d-old meristem sections, leaves, and stems (data not shown).

**DISCUSSION**

In these experiments, we have examined the mRNA accumulation of ALS genes in tobacco. ALS is an essential enzyme in branched-chain amino acid biosynthesis, and its sequence has been highly conserved, particularly among plant species (Mazur et al., 1987). ALS genes in plants may be single copy as in \textit{Arabidopsis} and sugarbeet species (Mazur et al., 1987; B.J. Mazur, unpublished data), or multicopy as in corn, soybean (S.J. Keeler, unpublished data), and tobacco (Mazur et al., 1987). Therefore, multiple isozymes of ALS do not appear to be required for the growth and development of plants, although it is possible that some species of plants maintain multiple isozymes of ALS for reasons related to organ-specific or developmental regulation.

Our results show that there is differential expression of ALS gene transcripts in several tobacco organs. ALS transcripts are expressed at a level of about 0.1% of mRNA, but the concentration of these messages can vary up to 4-fold in different organs. In situ hybridization studies show a ubiquitous expression of ALS message throughout most tissue types, with the greatest accumulation in metabolically active or rapidly dividing cell types of root, stem, and flowers. These findings suggest that the requirement for de novo production of branched-chain amino acids is essential to all plant cells, but that requirements may vary between organs and cell types. Younger organs such as seedlings, root tips, expanding leaves, and stem nodes may express higher levels of ALS message as part of a general mechanism for enhancing protein production in rapidly growing areas of the plant. Once development has progressed, as in older leaves, older roots, and stem sections without nodes, the need for protein precursors may decrease and the production of ALS transcripts can decline. Similarly, tissues such as the tapetum, embryo

<table>
<thead>
<tr>
<th>Organ</th>
<th>pg ALS Message/ ( \mu g ) Total RNA</th>
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<tr>
<td>Seedlings</td>
<td>10.5</td>
</tr>
<tr>
<td>Leaves 0–11 cm</td>
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<tr>
<td>Leaves 11–25 cm</td>
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<tr>
<td>Leaves &gt;25 cm</td>
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<td>Flowers</td>
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<tr>
<td>Roots</td>
<td>6.8</td>
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</table>

Table II. Quantitation of ALS gene mRNA levels in tobacco organs

Two-microgram aliquots of RNA were denatured and bound to nylon membranes using a 72-well dot-blot apparatus. Samples were hybridized to a labeled 2200-base anti-mRNA probe produced from plasmid pTZALS.1 that hybridized to both tobacco ALS mRNAs. A dilution series of in vitro-generated \textit{SurA} and \textit{SurB} gene transcripts was also bound to the membrane. The radioactivity associated with each sample was quantitated by scintillation counting. A standard curve was generated from the hybridization data for the control, unlabeled, in vitro \textit{SurA} and \textit{SurB} gene transcripts. The number of picograms of ALS message present per microgram of total RNA in each sample was then determined by comparison to the standard curve.

![Figure 4. In situ localizations of ALS gene expression in tobacco ovaries. Ovaries were fixed, embedded in paraffin, sectioned to 10-\(\mu\)m thickness, hybridized with either \(^{35}\)S-labeled anti-ALS mRNA or \(^{35}\)S-labeled sense-rRNA mRNA and subjected to autoradiography. A, Bright-field photograph of stage 12 ovary cross-section (\(\times10\)): W, wall; L, locule; O, ovule; P, placenta; V, vascular bundle. B, In situ hybridization of an anti-ALS mRNA probe with the section seen in A. White grains represent RNA/RNA hybridization. Photographs were taken with dark-field microscopy and slide exposure time was 10 d. C, In situ hybridization of anti-rRNA mRNA probe with stage 12 ovary cross-section (\(\times10\)). White grains represent background hybridization levels. Photographs were taken with dark-field microscopy and slide exposure time was 4 d.](www.plantphysiol.org)
Figure 5. Determination of the transcription initiation sites for the tobacco ALS genes. mRNAs synthesized from in vitro reactions or extracted from plants were annealed to 32P-labeled anti-mRNA probes. After RNase digestion, the protected, labeled fragments were separated on a 6% denaturing acrylamide gel. RNA and DNA standards were used to determine the size of the fragments. A, Determination of the transcription initiation site for the tobacco SurA gene. A 588-base SurA gene anti-mRNA probe produced from pTSNTE was annealed to: lane 1, 10 µg of tobacco total RNA; lane 3, Arabidopsis ALS in vitro mRNA; lane 4, tobacco SurA in vitro mRNA; lane 5, tobacco SurB in vitro mRNA. Lane 2 shows the undigested probe. In lane 4, the 538-base RNase digestion product corresponds to that predicted for hybridization of the SurA probe to the complementary SurA in vitro transcripts. B, Determination of the transcription initiation site for the tobacco SurB gene. A 520-base SurB gene anti-mRNA probe produced from plasmid pTSNTE was annealed to: lane 1, 10 µg of tobacco total RNA; lane 3, Arabidopsis ALS in vitro mRNA; lane 4, tobacco SurA in vitro mRNA; lane 5, tobacco SurB in vitro mRNA. Lane 2 shows the undigested probe. In lane 5, the 487-base RNase digestion product corresponds to that predicted for hybridization of the SurB probe to the complementary SurB in vitro transcripts.

sacs, and placenta may require a higher expression of transcripts for biosynthetic enzymes.

In all of the organ types and developmental stages studied here, the relative expression of the two tobacco ALS genes is coordinately modulated. For example, in seedlings where there is a relatively high level of SurA transcript, there is a coordinately higher level of SurB transcript, such that a constant ratio of gene expression is maintained. Moreover, the pattern of transcriptional start sites visualized in RNase protection experiments is the same in all organs examined. The coordinate modulation of these genes in all organs examined suggests a common regulatory mechanism. These results indicate that the two genes are functionally redundant and are maintained because of the tetraploid nature of N. tabacum. However, it is possible that the two genes may be expressed in a cell-type-specific manner. To determine if there is any cell-type-specific expression of the two ALS gene transcripts, in situ hybridizations utilizing transcript-specific probes would be required. Because of the extensive homology between the genes, we have not found oligonucleotide probe sequences that allow differentiation of in vitro-generated sense transcripts on RNA dot blots (data not shown). Alternatively, fusing the ALS promoters to a reporter gene such as that encoding β-glucuronidase could permit cell-type-specific expression to be assayed in transgenic tobacco lines.

The sequence specificity responsible for the greater steady-state SurB message level has not been determined. Both tobacco ALS genes have highly homologous sequences in their noncoding regions, from —1 to —170 (Fig. 6). There is one major start site at —60 for the SurB gene, and three possible starts at —54 to —63 for the SurA gene. There are 12 mismatches in the region that lies between the proposed CAAT box, near —130, and the proposed TATA box, near —70. In the CAAT box, two bases differ between the sequences, and in the TATA box, there is a single A for T transversion. This latter single-base change makes the SurB promoter more homologous to the consensus sequence for plant promoters proposed by Messing et al. (1983), and could affect the transcriptional efficiency of this promoter. In fact, the increased accumulation of the SurB transcript over the SurA transcript correlates with observed differences in the transformation efficiency of herbicide-resistant forms of these genes; transformation of tobacco cells is more efficient with SurB genes than with SurA genes (B.J. Mazur, unpublished results).

The transcriptional regulation observed for the tobacco ALS genes differs from that found in other amino acid pathway multigene families, such as the glutamine synthetase genes. In Pisum sativum, particular glutamine synthetase gene products are expressed in a nonoverlapping, cell-specific pattern, such that one gene is expressed only in photosynthetic cell types and another gene only in vascular cell types (Edwards et al., 1990). In Phaseolus vulgaris, the multigene family of glutamine synthetase genes appear to be differen-

Figure 6. Sequences flanking ALS gene transcription initiation sites. Comparison of the 5’ regions of the tobacco SurA and SurB genes. Dots indicate identical nucleotides in the two genes. Dashes represent insertions/deletions that have been placed to facilitate alignment of the two sequences. All numbers refer to the “A” of the ATG translational start codon as +1; the numbers shown are for the consensus sequence. Boxes denote possible CAAT and TATA transcription regulatory sequences as defined by Messing et al. (1983). The arrows denote the transcription start sites of each gene as determined from the RNase protection experiments shown in
Regulation of Acetolactate Synthase Genes

(A) 588
(B) 520
517
506
417(A)

Figure 7. Relative ratios of SurA and SurB gene transcripts in tobacco organs. RNase protection analyses were used to determine the relative proportion of SurA and SurB gene transcripts in different tobacco organ types. Ten-microgram samples of total RNA from tobacco leaves (lanes 1 and 2), stems (lanes 3 and 4), roots (lanes 5 and 6), and flowers (lanes 7 and 8) were annealed with either a SurA anti-mRNA probe (designated as A) or a SurB anti-mRNA probe (designated as B). After RNase digestion, the labeled, protected fragments were separated on a 6% denaturing acrylamide gel. Only the upper portion of the gel is shown in the photograph. Undigested probes (lanes 10 and 11) and labeled size standards (lane 9) were used to determine the lengths of the protected fragments. Primary digestion products migrated at about 417 bases for the SurA (A) probe and 409 bases for the SurB (B) probe. The roots used in this experiment were grown in vermiculite rather than sand, which may have reduced the recovery of the rapidly growing root hairs and the amount of ALS message compared with that in Figure 1 and Table II.

Primarily expressed in developing leaves (Cock et al., 1991). The regulation of ALS genes also differs from the regulation determined for the gene coding for EPSPS, an enzyme involved in the synthesis of aromatic amino acids in plants. EPSPS message is differentially expressed in petunia, where the relative proportion of SurA and SurB gene transcripts in different tomato organ types. Ten-microgram samples of total RNA from tobacco leaves (lanes 1 and 2), stems (lanes 3 and 4), roots (lanes 5 and 6), and flowers (lanes 7 and 8) were annealed with either a SurA anti-mRNA probe (designated as A) or a SurB anti-mRNA probe (designated as B). After RNase digestion, the labeled, protected fragments were separated on a 6% denaturing acrylamide gel. Only the upper portion of the gel is shown in the photograph. Undigested probes (lanes 10 and 11) and labeled size standards (lane 9) were used to determine the lengths of the protected fragments. Primary digestion products migrated at about 417 bases for the SurA (A) probe and 409 bases for the SurB (B) probe. The roots used in this experiment were grown in vermiculite rather than sand, which may have reduced the recovery of the rapidly growing root hairs and the amount of ALS message compared with that in Figure 1 and Table II.

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Regulation of ALS genes has also been studied in other plant species, such as soybean, where the level of the EPSPS transcript is similar in all organs observed (Gasser et al., 1988). The amino acid biosynthetic gene family for Asn synthetase is differentially regulated in response to light (Tsai and Coruzzi, 1990). We have not investigated light regulation of the ALS genes, but because both genes are expressed in all organs and tissues examined, including roots, it is unlikely that ALS genes are light regulated.

Figure 7. Relative ratios of SurA and SurB gene transcripts in tobacco organs. RNase protection analyses were used to determine the relative proportion of SurA and SurB gene transcripts in different tobacco organ types. Ten-microgram samples of total RNA from tobacco leaves (lanes 1 and 2), stems (lanes 3 and 4), roots (lanes 5 and 6), and flowers (lanes 7 and 8) were annealed with either a SurA anti-mRNA probe (designated as A) or a SurB anti-mRNA probe (designated as B). After RNase digestion, the labeled, protected fragments were separated on a 6% denaturing acrylamide gel. Only the upper portion of the gel is shown in the photograph. Undigested probes (lanes 10 and 11) and labeled size standards (lane 9) were used to determine the lengths of the protected fragments. Primary digestion products migrated at about 417 bases for the SurA (A) probe and 409 bases for the SurB (B) probe. The roots used in this experiment were grown in vermiculite rather than sand, which may have reduced the recovery of the rapidly growing root hairs and the amount of ALS message compared with that in Figure 1 and Table II.

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LITERATURE CITED

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