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Tissue Culture-Specific Expression of a Naturally Occurring Tobacco Feedback-Insensitive Anthranilate Synthase[1]

Hee-Sook Song, Jeffrey E. Brotherton, Robert A. Gonzales, and Jack M. Widholm*

Department of Crop Sciences, University of Illinois, Edward R. Madigan Laboratory, 1201 West Gregory, Urbana, Illinois 61801 (H.-S.S., J.E.B., J.M.W.); and The Samuel Roberts Noble Foundation, Inc., P.O. Box 2180, 2510 Sam Noble Parkway, Ardmore, Oklahoma 73402 (R.A.G.)

A cDNA and corresponding promoter region for a naturally occurring, feedback-insensitive anthranilate synthase (AS) α-subunit gene, *ASA2*, has been isolated from an unselected, but 5-methyl-tryptophan-resistant (5MT^r), tobacco (*Nicotiana tabacum*) cell line (AB15–12-1). The *ASA2* cDNA contains a putative transit peptide sequence, and Southern hybridization shows that more than one closely related sequence is present in the tobacco genome. The *ASA2* cDNA complemented a *trpE* nonsense mutant *Escherichia coli* strain, allowing growth on 300 μM 5MT-containing minimal medium without tryptophan, and cell extracts contained feedback-insensitive AS activity. The 5MT^r was lost when the *E. coli* strain was transformed with an *ASA2* site-directed mutant (phenylalanine-107-arginine-108 → serine-107-glutamine-108). Identical nucleotide sequences encoding the phenylalanine-107-arginine-108 region have been found in polymerase chain reaction-amplified 326-bp *ASA2* genomic fragments of wild-type (5-methyl-tryptophan-sensitive [5MT^s]) tobacco and a progenitor species. High-level *ASA2* transcriptional expression was detected only in 5MT^r-cultured cells, not in 5MT^s cells or in plants. Promoter studies indicate that tissue specificity of *ASA2* is controlled by the promoter region between −2252 and −607. Since the *ASA2* promoter sequences are not substantially different in the 5MT^r and 5MT^s lines, the increased levels of *ASA2* mRNA in the 5MT^r lines are most likely due to changes in a regulatory gene affecting *ASA2* expression.

Many aromatic compounds are synthesized via the shikimate pathway in higher plants (Haslam, 1993; Herrmann, 1995). Chorismate is located at a branch point of this pathway and is the last common precursor of many of these compounds. AS catalyzes the first committed reaction in the Trp-biosynthesis branch by converting chorismate to anthranilate and is feedback inhibited by the end product, Trp (Haslam, 1993; Romero et al., 1995). That AS is the control point in the Trp branch in plant cells is indicated by (a) pathway-intermediate feeding studies (Widholm, 1974), (b) enzyme activity levels, (c) feedback inhibition of the respective enzyme activities (Singh and Widholm, 1974), and (d) 5MT resistance selection that yielded lines with altered feedback-inhibited AS and higher free Trp (Widholm, 1972a, 1972b).

Plant cell culture systems have been useful for studying the regulatory mechanisms of amino acid biosynthesis (Widholm, 1972a, 1972b). Selecting cells resistant to amino acid analogs such as 5MT can produce lines with increased amounts of the end product, Trp, because of an alteration of the allosteric regulatory enzyme, causing less inhibition of the enzyme by the analog and Trp (Widholm, 1972a, 1972b). Feedback-insensitive AS has been found in 5MT-selected carrot (*Daucus carota*; Brotherton et al., 1986), *Datura innoxia* (Ranch et al., 1983), potato (*Solanum tuberosum*; Carlson and Widholm, 1978), and tobacco (*Nicotiana tabacum*; Brotherton et al., 1986). The 5MT^r tobacco cell line (TX2–4) contained Trp-insensitive AS and higher levels of free Trp than wild-type cells. Similar results were obtained with 5MT-selected cultured potato cells (Carlson and Widholm, 1978). These characteristics were not found in the regenerated tobacco plants but were recovered again in cultured cells induced from leaves of the regenerants (Brotherton et al., 1986). These results suggest that expression of this altered AS is regulated in a tissue-specific manner and that selection for 5MT^r produces lines with increased amounts of the feedback-insensitive AS form that is expressed in cultured cells but not in plants.

Recently, AS was purified from cultured plant cells or plant tissues (Poulsen et al., 1993; Bohlmann et al., 1995; Romero and Roberts, 1996) and found to have a subunit composition similar to AS from some microbes, i.e. nonidentical large (α, component I) and small (β, component II) subunits (Zalkin et al., 1984; Yanofsky and Crawford, 1987; Crawford, 1989). AS genes encoding an α-subunit (Niyogi and Fink, 1992; Bohlmann et al., 1995) and β-subunit (Niyogi et al., 1993) have been cloned from Arabidopsis and *Ruta graveolens*. The two AS genes encoding the α-subunit of the enzyme cloned from Arabidopsis and *R. graveolens* have been designated *ASA1*/*ASA2* and *ASα1*/*ASα2*, respectively (Niyogi and Fink, 1992; Bohlmann et al., 1995). These AS genes have similar gene-expression patterns, since *ASA1* and *ASα1* expression is induced by wounding and/or elicitor treatment, whereas the *ASA2* and *ASα2* genes are expressed constitutively at low levels (Niyogi and Fink, 1992; Bohlmann et al., 1995).

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* Corresponding author; e-mail widholm@uiuc.edu; fax 1–217–333–4777.

Abbreviations: AS, anthranilate synthase; CaMV, cauliflower mosaic virus; IPTG, isopropylthiogalactoside; MS, Murashige-Skoog; 5MT, 5-methyl-Trp; 5MT^r, 5-methyl-Trp-resistant; 5MT^s, 5-methyl-Trp-sensitive; MUG, 4-methylumbelliferone glucuronide; RACE, rapid amplification of cDNA ends; UTR, untranslated region.

Two groups have described a mutant Arabidopsis AS gene encoding a feedback-insensitive α-subunit of the enzyme (Kreps et al., 1996; Li and Last, 1996). Sequencing of these mutant AS genes showed that a single amino acid, Asp-341, was replaced by Asn in both cases. These mutant AS genes were expressed in both cultured cells and leaves, with slightly higher expression in cultured cells (Kreps et al., 1996). In contrast to these Arabidopsis mutants, the *ASα1* gene from *R. graveolens* that encodes a feedback-insensitive α-subunit was isolated from wild-type young shoots (Bohlmann et al., 1996). Based on sequence comparison, it was postulated that a single amino acid substitution in the *ASα1* gene, Arg-138 for Gln-138, may cause feedback insensitivity. The residues potentially affecting feedback inhibition in higher plants do not align with those of bacteria (Matsui et al., 1987; Caliguri and Bauerle, 1991) and yeast (Graf et al., 1993), but the altered amino acids are located within or very close to the conserved motifs Leu-Leu-Glu-Ser-X_{10}-Ser and Asn-Pro-Ser-Pro-Tyr-Met, which are important for feedback inhibition (Matsui et al., 1987; Caliguri and Bauerle, 1991; Graf et al., 1993).

The tobacco plants regenerated from the $5MT^r$ suspension cultures described by Brotherton et al. (1986) were not fertile; therefore, the selection was repeated with new cultures to obtain fertile plants (S. Schechter and J.M. Widholm, unpublished data). One of nine plants regenerated from an unselected control culture produced a suspension culture from a leaf that was $5MT^r$. This resistance was inherited by progeny, and the cell line studied here, AB15–12-1, was initiated from a fifth-generation plant. This cell line has the typical characteristics of $5MT^r$ cells, feedback-insensitive AS and increased free Trp. We report the cloning and characterization of the wild-type tobacco *ASA2* gene encoding a feedback-insensitive α-subunit of the enzyme and analysis of the role of promoter regions in tissue-specific expression of this gene. We show that *ASA2* mRNA is specifically elevated in $5MT^r$ lines and propose that this increased level of expression is due to enhanced transcription of the *ASA2* gene in the $5MT^r$ lines.

MATERIALS AND METHODS

Suspension cultures were maintained by weekly transfers into 50 mL of liquid MX medium (MS basal medium [Murashige and Skoog, 1962] with 1.8 μM 2,4-D). The $5MT^r$ AB15–12-1 cell line originated from a tobacco (*Nicotiana tabacum* cv Xanthi) plant regenerated from unselected suspension-cultured cells. Originally, nine plants were regenerated by placing the unselected cells on MS agar-solidified medium with 5.7 μM IAA and 2 μM kinetin. Callus and subsequent suspension cultures were initiated from the leaves of these plants. One plant produced a suspension culture that showed resistance to 5MT when 0.5 g fresh weight of cells was incubated for 9 to 12 d in MX liquid medium containing 46 and 137 μM 5MT; other wild-type cells did not grow in the same medium. Because this plant was male-sterile, it was pollinated with wild-type pollen and the resulting progeny were self-pollinated four times to obtain AB15–12-1. Additional tobacco cell-suspension cultures were subsequently initiated from AB15–12-1 seedlings and maintained on MX or 5MT-containing medium as indicated.

Nucleic Acid Analysis

Genomic DNA was isolated from 1-week-old suspension-cultured cells using the procedures of Dellaporta et al. (1983) and purified by CsCl-gradient centrifugation (Ausubel et al., 1989; Sambrook et al., 1989).

Total RNA was prepared using a phenol-extraction method (Wang et al., 1994) from 1-week-old suspension-cultured cells, mature seeds, and leaves, roots, and stems harvested from 3-week-old shoot cultures grown on MS basal medium solidified with 0.8% agar.

DNA and RNA gels were blotted onto a nylon membrane (Hybond N^+, Amersham) following a general capillary-transfer method (Sambrook et al., 1989). The *Arabidopsis thaliana ASA1* (pKN41) cDNA clone (obtained from K. Niyogi [Niyogi and Fink, 1992]) or other tobacco AS cDNA clones obtained in this work were used as a probe following labeling with a Megaprime DNA-labeling system (Amersham) with [α-^{32}P]dCTP (3000 Ci/mmol). Southern and northern hybridizations were done at 42°C with hybridization solution (50% formamide, 5× SSPE, 5× Denhardt's solution [Sambrook et al., 1989], 0.1% SDS, and 100 μg/mL salmon-sperm DNA). The membranes were washed at high stringency twice at room temperature with 2× SSC and 0.5% SDS for 20 min each time and at 65°C with 0.1× SSC and 0.1% SDS until the background signal disappeared and were then exposed to radiographic film overnight with an intensifying screen.

Cloning of AS cDNA

Tobacco AS cDNAs were isolated using 5′ and 3′ RACE (GIBCO-BRL). Primers 1 and 2 for cloning the 5′ end of the *ASA2* cDNA were designed based on the sequence of the Arabidopsis *ASA1* genomic clone (GenBank accession no. M92353). The sequences of primers used for cloning are listed in Table I. For 5′ RACE, first-strand cDNA was synthesized with antisense primer 1 at nucleotide position 5403 to 5382 of the Arabidopsis *ASA1* genomic clone. The 5′ end of the first-strand cDNA was oligo-dA tailed (200 μM dATP) using terminal deoxynucleotidyl transferase (0.4 unit/μL).

A nested PCR was performed with the adapter primer 5′-GGCCACGCGTCGACTAGTAC$(T)_{17}$-3′ (GIBCO-BRL) and antisense primer 2 at nucleotides 4430 to 4409 of the Arabidopsis *ASA1* genomic clone. The first-strand cDNA was used as a template for the nested PCR. A fragment (approximately 1.1 kb) was amplified and cloned into the pGEM-T vector and sequenced. The procedures used to isolate the 3′ end of the *ASA2* cDNA were the same as for 5′ RACE except for the primers and dATP tailing at the 5′ end of the cDNA. Primer 3 was designed based on the sequence of the 5′ *ASA2* cDNA clone at nucleotides +148 to +168 (adenine of ATG was counted as +1, since the transcription start site was not determined in this work). An approximately 1.9-kb fragment was amplified and strongly hybrid-

Table I. *Nucleotide sequences of the primers used for cloning*

Restriction enzyme site for cloning at 5′ end of the primers is underlined. Boldfaced nucleotides in the primers 7 and 8 represent mismatch nucleotides for site-directed mutagenesis.

Primer	Sequence	Location[a]
1[b]	5′-GCGGCTTTGTTCTGGCACTCA-3′	
2[b]	5′-CTGCAAATGTTCGCCGCTCAA-3′	
3	5′-CTAGTTATGGATGAGGACAGG-3′	+148/+168
4	5′-ACTAGTGGATCCTCTAAAAGCGGGAACTTG	+181/+198
5	5′-TTGCGGGGTACCCTAGTTTCTTTTCTCATGTAC-3′	+1851/+1831
6	5′-ACTAGTGGATCCTGCCTTCACTCTTCATCTCTAG-3′	+130/+151
7	5′-ACCTTGAGACCCGGGTTCAACGGATTCAAAGAGAAAGCTTGG-3′	+327/+286
8	5′-TCCGTTGAACCCGGGTCTCAAGGTTCTAGTGTTGGTCGCTAC-3′	+304/+345
9	5′-GATCCCATTTTCAAAGTCACC-3′	−110/−90
10	5′-TGTTCCTTAGCCACAATTTCC-3′	+392/+372
11	5′-ACGACTGCATTCCTACAAGAG-3′	+10/−11
12	5′-GGATCCCCCGGGTCCTACAAGAGCACAATA3′	−1/−18
13	5′-GCATGCCTGCAGCAAATCTATTCGATAGTG-3′	−2252/−2235
14	5′-GCATGCCTGCAGTAGGCAATACGGCACATA-3′	−1356/−1339
15	5′-GCATGCCTGCAGTGTATTGCCCATTTCATT-3′	−606/−589
16	5′-GCATGCCTGCAGTCAGCCAAATGTGTCCAA-3′	−370/−353

[a] Locations of primers were numbered based on the nucleotide sequence. Adenine of the translation start codon (ATG) was numbered as a +1. [b] Degenerated primers 1 and 2 were designed based on the sequence of the Arabidopsis *ASA1* genomic clone.

ized with the 5′ *ASA2* cDNA clone as a probe. This fragment was cloned into the pGEM-T vector and sequenced.

Analysis of the sequence alignment of the two fragments using Clustal (Higgins and Sharp, 1989) shows that the 828-bp overlapping region is identical. There is only one *Xba*I site in the 828-bp overlapping region, only one *Nsi*I site in the pGEM-T vector, and no *Nsi*I site in either the 5′ or the 3′ fragments. These two restriction enzyme sites were used to construct the full-length tobacco *ASA2* cDNA. Sequencing of the cDNA clones was performed by the Genetic Engineering Laboratory of the University of Illinois (Urbana-Champaign) using the dideoxynucleotide-sequencing method.

Complementation Tests and Site-Directed Mutagenesis

The *ASA2* cDNA without the presumed transit-peptide sequence (amino acid sequence 1–60) was amplified using *Pfu* DNA polymerase (Stratagene) with primers 4 and 5 (Table I) containing *Bam*HI and *Kpn*I overhangs, respectively, and ligated into an expression vector (pQE30 containing a 6-His tag-coding sequence [6xHis, Qiagen, Santa Clarita, CA]) followed by restriction-enzyme digestion.

Site-directed mutagenesis was performed by PCR using a primer containing mismatched nucleotide sequences by changing four nucleotides (CCTGGTTTTCGA→CCCGGGTCTCAA) at amino acid residues 105 to 108. The first two mismatched nucleotides, Pro-105 and Gly-106, do not change the amino acid codon but create an *Sma*I site. The last two mismatched nucleotides change Phe-107 and Arg-108 to Ser-107 and Gln-108. Two PCR products were obtained using primers 6 and 7 and primers 8 and 5 (Table I). These two PCR fragments were ligated, digested by *Sma*I, and ligated in-frame into the pQE30 vector.

The chimeric constructs were transformed into *trpE* mutant *Escherichia coli* (*trpE5972*, nonsense mutant) using $CaCl_2$ transformation (Sambrook et al., 1989). Complemented strains were plated on M9 minimal medium containing 100 μg/mL ampicillin and 0.1 mM IPTG but no Trp. For the inhibition test, 300 μM 5MT was added to the minimal medium described previously.

Expression of 6xHis-Tagged ASA2 Protein in *E. coli*

The *E. coli trpE* mutant (*trpE5972*) transformed with the 6xHis-tagged *ASA2* chimeric construct was grown in 1 L of Luria-Bertani medium supplemented with 100 μg mL^{-1} ampicillin and 0.1 mM Trp. IPTG (0.1 mM) and PMSF (0.1 mM) were added to a mid-logarithm culture. After 3 h of further incubation at 30°C and 150 rpm, the cells were harvested by centrifugation, resuspended in 20 mL of extraction buffer (Bernasconi et al., 1994) with 0.1 mM PMSF added, disrupted using a French press (two passages at 20,000 p.s.i.), and centrifuged to remove cell debris. The supernatant was held overnight on ice and then combined with 2 volumes of saturated, room-temperature $(NH_4)_2SO_4$. The resulting protein precipitate was collected by centrifugation at 4°C and resuspended in 10 mL of 50 mM NaH_2PO_4, 300 mM NaCl, and 2 mM DTT, pH 8.0. The 6xHis-tagged ASA2 protein was partially purified after binding to Ni-nitrilotriacetic acid resin (Qiagen) and elution with 100 mM imidazole in 50 mM NaH_2PO_4, 300 mM NaCl, 2 mM DTT, and 10% glycerol, pH 6.0.

Arabidopsis ASA1 protein fused with glutathione *S*-transferase was expressed using the pSCI1674 construct cloned into *E. coli* MC1061 by Bernasconi et al. (1994). The procedure was the same as described above except that the resuspended $(NH_4)_2SO_4$ pellet was used without further purification.

AS Enzyme Assay

Cell extracts were prepared as described by Brotherton et al. (1986) from mid-logarithm-phase cell cultures and were

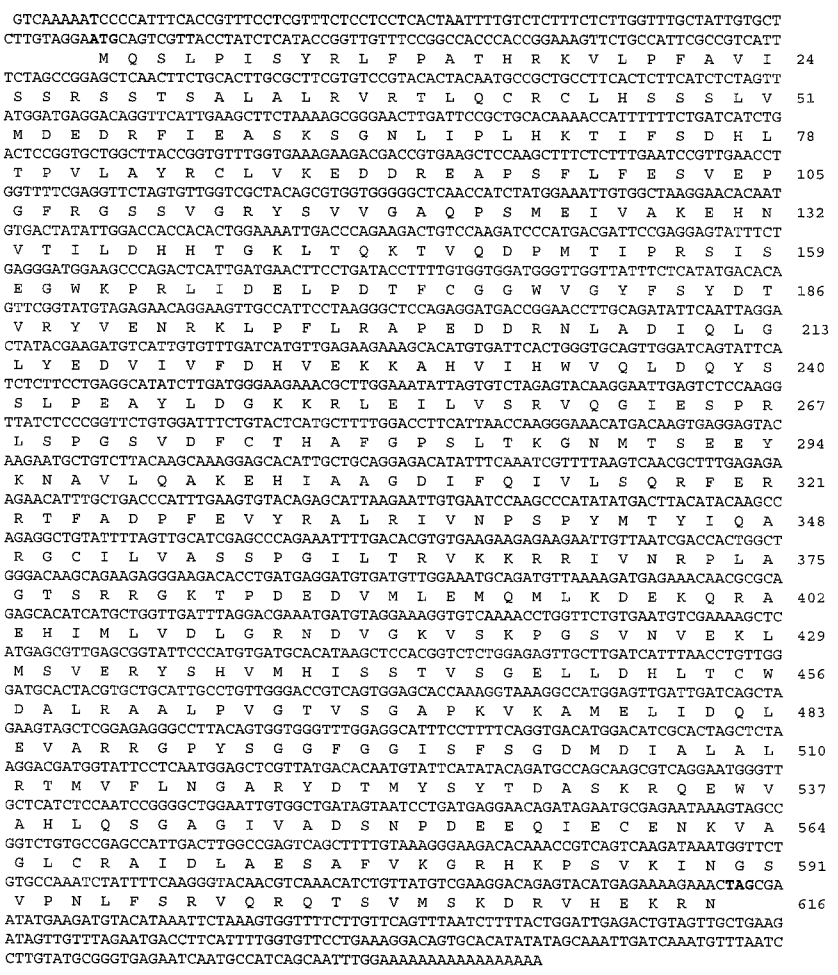

Figure 1. Nucleotide and the predicted amino acid sequence of the *ASA2* cDNA. The coding region corresponds to amino acid sequence 1 (ATG) to 616 (TAG). The putative translation start and stop codons are indicated in boldface. The 89 and 205 bp of the upstream and downstream coding region represent 5′ and 3′ UTRs, respectively.

used the same day. The resuspended $(NH_4)_2SO_4$ fraction was desalted using Sephadex G-25.

AS activity was measured using the ethyl-acetate-extraction method used previously for tobacco cell extracts (Widholm, 1971) and incorporating the modifications described by Li and Last (1996), except that the assay buffer was 50 mM Hepes, pH 7.5, 10 mM Gln, 2.0 mM $MgCl_2$, 0.05 mM Na_2EDTA, 2.0 mM DTT, and 5% glycerol. The reaction was started by the addition of 50 μL of cell extract. Chorismate and Trp concentrations were as indicated in individual experiments. The production of anthranilate was linear with respect to time for twice the standard incubation time and enzyme concentration (data not shown). The conversion of chorismate to anthranilate never exceeded 1% in any experiment. Chorismate was produced using the fermentation method of Gibson (1970).

ASA2 Genomic Fragments Containing the Phe-107-Arg-108 Region from Wild-Type *Nicotiana* sp.

ASA2 genomic fragments containing the Phe-108-Arg-108 region were PCR amplified and isolated from three wild-type ($5MT^s$) cultured cell lines, *N. tabacum* (TXD), *Nicotiana tomentosiformis* (Nto), and *Nicotiana sylvestris* (Ns), from leaves harvested from AB15–12-1 shoot cultures, and from two $5MT^s$ (H15–6, NRMX) and one $5MT^r$ (NR5MT) tobacco cell lines. The region from −110 to +392 was first amplified using two sets of primers based on the *ASA2* cDNA and promoter sequences 9 and 10 (Table I). Primers 4 and 10 were then used for a nested PCR amplification using the first PCR product as the template to produce the 326-bp genomic fragments (+181 to +392) that were then cloned into pBluescriptSK−, digested with *Eco*RV, and sequenced.

Cloning of AS Promoter and Construction of Chimeric AS Promoter-GUS Constructs

The *ASA2* promoter was isolated using inverse PCR (Ochman et al., 1989). AB15–12-1 genomic DNA was digested with *Hin*dIII, circularized with T4 DNA ligase after dilution to 100 ng/mL with distilled, deionized water and used as a template for inverse PCR with primer 3 and antisense primer 11 based on the sequence of the *ASA2* cDNA at nucleotide +10 to −11. PCR amplification was performed for 30 cycles (95°C, 1 min; 50°C, 40 s; 72°C, 2

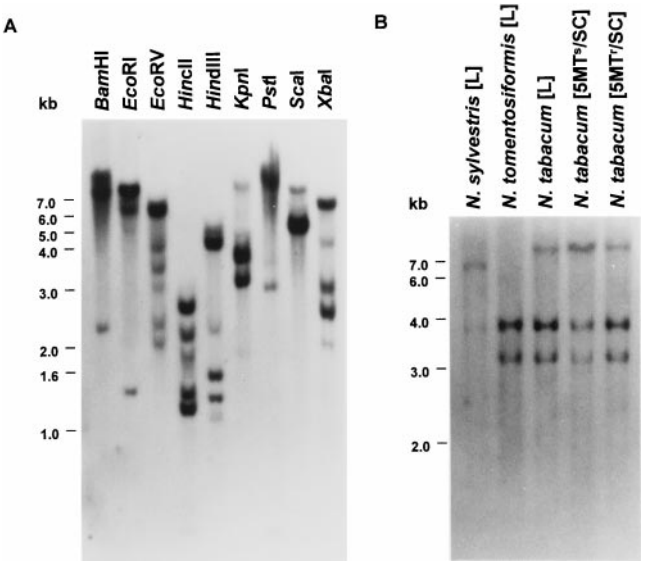

Figure 2. Genomic DNA-blot analysis. A, Twenty micrograms of AB15–12-1 genomic DNA was digested with nine restriction enzymes. Lanes from left to right correspond to *Bam*HI, *Eco*RI, *Eco*RV, *Hinc*II, *Hind*III, *Kpn*I, *Pst*I, *Sca*I, and *Xba*I. B, Ten micrograms of each genomic DNA obtained from leaves of *N. sylvestris*, *N. tomentosiformis*, *N. tabacum*, and suspension-cultured cells of the $5MT^s$ and $5MT^r$ tobacco cell line (AB15–12-1) was digested with *Kpn*I. The full-length *ASA2* cDNA clone was used as a probe in both A and B. Fragment sizes were determined by a 1-kb ladder (GIBCO-BRL).

min). A PCR fragment (approximately 2.3 kb) was detected by Southern hybridization with the *ASA2* cDNA as a probe and cloned into pGEM-T vector. Sequencing of the *ASA2* promoter (approximately 2.3 kb) was performed at the Molecular Analysis and Synthesis Section of the Samuel Roberts Noble Foundation (Ardmore, OK) using the dideoxynucleotide-sequencing method.

Based on database search results, deletions were made by using PCR amplification with seven sets of primers as follows: antisense primer 12 with sense primers 13, 14, 15, or 16 (Table I) that amplified 2252-, 1356-, 606-, or 370-bp ASA2 promoter fragments (including the 5′ UTR), respectively. These PCR products were fused to the GUS reporter gene with NOS terminator. The chimeric constructs were designated 2252, 1356, 606, and 370. Each primer contains a restriction enzyme site overhang for cloning. Primer 12 contains an *Sma*I site and primers 13, 14, 15, and 16 contain a *Pst*I site. Each restriction enzyme site was underlined in the primer sequences (Table I). These five fragments were cloned into pBI221, replacing the CaMV 35S promoter.

GUS Assay

The constructed plasmid DNAs were isolated using a Plasmid Maxi Kit (Qiagen) and biolistically transferred into tobacco suspension-cultured cells (AB15–12-1) and leaves using a particle-inflow gun (5 μg of DNA and 0.5 mg of 1.0-μm diameter gold particles [Bio-Rad] shot at 80 p.s.i.; Vain et al., 1993). Leaves (180–350 mg fresh weight) har-

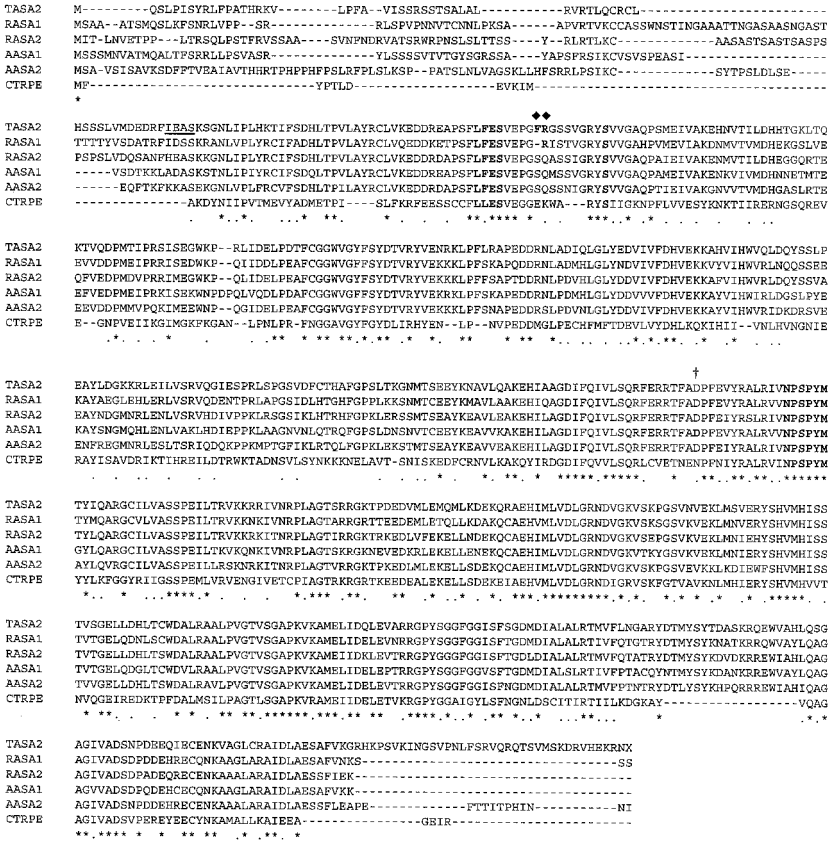

Figure 3. Amino acid sequence alignment of AS genes from plants and a prokaryote. TASA2, RASA1, RASA2, AASA1, AASA2, and CTRPE correspond to *N. tabacum ASA2*, *R. graveolens ASα*1 and *ASα*2, *A. thaliana ASA1* and *ASA2*, and *C. thermocellum trpE* cDNA clones, respectively. Dashes within sequences indicate gaps. Asterisks under the sequence represent identical amino acids among these six different AS sequences. Dots under the sequence indicate similar amino acids. Two consensus motifs, Leu-Leu-Glu-Phe-X_{10}-Ser and Asn-Pro-Ser-Pro-Tyr-Met, affecting feedback inhibition based on microorganisms and yeast, are indicated in boldface. The amino acid(s) substitutions Phe-Arg and Arg, which caused feedback insensitivity in *N. tabacum* and *R. graveolens*, are indicated in boldface with a diamond mark at positions 107 and 108, respectively. A single amino acid change in an Arabidopsis mutant (Asp to Asn) is indicated in boldface with a dagger at position 326. A consensus sequence motif for plant chloroplast transit peptides in the tobacco ASA2 is underlined at positions 58 to 61. The amino acid numbers indicated are based on the tobacco *ASA2* amino acid sequence.

vested from 3-week-old shoot cultures were placed on 5-cm-diameter sterile Whatman no. 1 filter paper discs. Five milliliters of 2-d-old AB15–12-1 suspension-cultured cells (approximately 200 mg fresh weight) was collected on filter paper using a vacuum before bombardment. The samples were transferred with the filter paper onto MX solid medium after bombardment and incubated at 24°C under fluorescent light (60 μE m^{-2} s^{-1}) for 3 d. The promoter activity was determined with the fluorometric MUG assay (Jefferson, 1987; Jefferson et al., 1987). GUS-specific activity was determined as picomoles of 4-methylumbelliferone per hour per milligram of protein. The protein concentration was determined using a protein dye-binding assay kit (Bio-Rad).

RESULTS

ASA2 cDNA Cloning and Sequence Analysis

We have cloned a full-length *ASA2* (2.16 kb including 5′ and 3′ UTR) cDNA using 5′ and 3′ RACE. The nucleotide and deduced amino acid sequences are shown in Figure 1. This AS gene is denoted *ASA2*, since its amino acid sequence was 72% identical to the sequence of *AS*α2 of *R. graveolens*. An apparent 5′ UTR was found 89 nucleotides upstream of a translation start codon (ATG) in the 5′ end of the cDNA fragment. The 3′ RACE results indicate that polyadenylation begins 205 nucleotides downstream of the translation stop codon. No perfect match to the consensus eukaryotic poly(A^+) signal sequence AAUAAA was found in the 3′ UTR.

The *ASA2* cDNA clone hybridized under high-stringency conditions to several fragments of genomic DNA digested with nine restriction enzymes (Fig. 2A). Three major bands at approximately 3.3, 4.0, and 24 kb were found in *Kpn*I-digested genomic DNA of the amphidiploid *N. tabacum* leaves and the two cultured cell lines, whereas the diploid tobacco progenitors, *N. sylvestris* and *N. tomentosiformis*, showed different patterns (Fig. 2B). The hybridization pattern of *N. tabacum* was more like *N. tomentosiformis* than *N. sylvestris*, since both had bands at approximately 3.3 and 4.0 kb. Two faint bands hybridized to the *ASA2* cDNA clone in *N. sylvestris*. The 24-kb fragment in *N. tabacum* shows very weak hybridization and is not found in either *N. sylvestris* or *N. tomentosiformis*.

Comparison of Amino Acid Sequences of AS Genes

Based on BLAST analysis (Altschul et al., 1990), the five best matches to the predicted amino acid sequence of the tobacco *ASA2* gene are aligned in Figure 3. The 1851 nucleotide *ASA2*-coding region would encode a 616-amino acid polypeptide with a calculated molecular mass of 69,035 D. The tobacco *ASA2* gene encodes 60 amino acids beyond the amino terminus of the aligned microbial homologs. The sequence Ile-58-Glu-59-Ala-60-Ser-61 is similar to the consensus cleavage-site motif ([Val/Ile]X[Ala/Cys] ↓ Ala) for plant chloroplast transit peptides (von Heijne et al., 1989; Gavel and von Heijne, 1990). The transit peptide sequences of the five plant AS genes show little homology to each other. The tobacco *ASA2* gene shows 72 and 70, 67 and 66, and 32% amino acid identity to *R. graveolens AS*α2 and *AS*α1, *A. thaliana* ASA1 and *ASA2*, and *Clostridium thermocellum trpE* genes, respectively (Sato et al., 1989; Niyogi and Fink, 1992; Niyogi et al., 1993; Bohlmann et al., 1995).

In bacteria, fungi, and yeast two conserved amino acid motifs, Leu-Leu-Glu-Ser-X_{10}-Ser and Asn-Pro-Ser-Pro-Tyr-Met, are associated with Trp feedback inhibition (Matsui et al., 1987; Caliguri and Bauerle, 1991; Graf et al., 1993). The six AS genes in Figure 3 showed no amino acid changes in these conserved regions except in the Leu-Leu-Glu-Ser-X_{10}-Ser motif, where the Ser and Gln residues found in

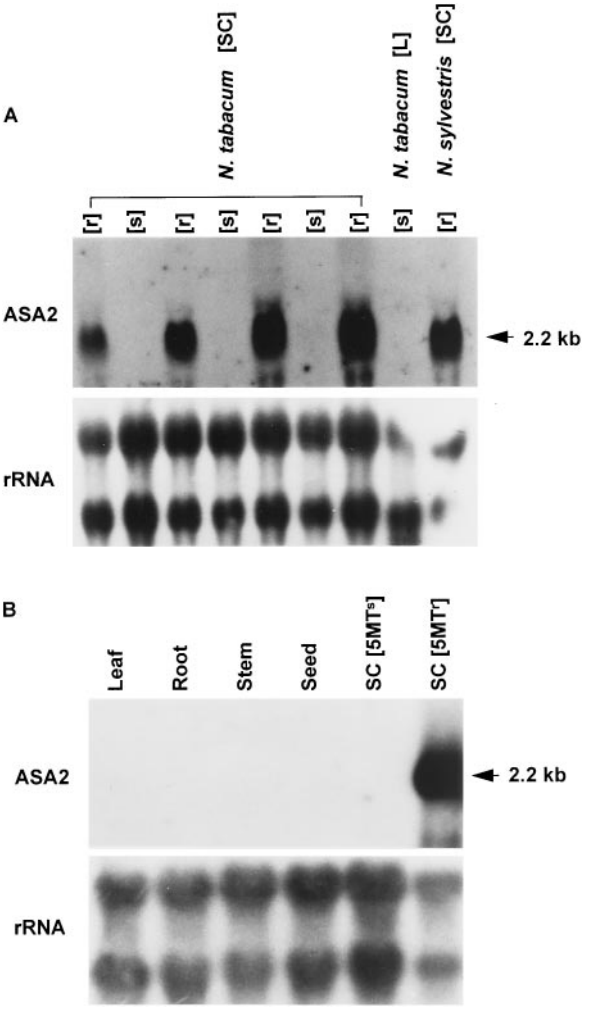

Figure 4. Northern analysis. Twenty micrograms of total RNA per lane was used for northern hybridization. A full-length *ASA2* cDNA clone (ASA2) and rRNA (rRNA) were used as probes for hybridization. A, Total RNAs were prepared from 1-week-old *N. tabacum* suspension-cultured cells (SC) of four $5MT^s$ (s) and four $5MT^r$ (r) cell lines, $5MT^s$ *N. tabacum* leaves (L) harvested from 3-week-old AB15–12-1 shoot cultures, and 1-week-old $5MT^r$ *N. sylvestris* suspension-cultured cells. B, Different plant organs were used to determine tissue specificity. AB15–12-1 leaves, roots, and stems harvested from 3-week-old shoot cultures and dried mature seeds and suspension-cultured $5MT^s$ cells (TXD) and $5MT^r$ cells (AB15–12-1) were used to prepare total RNA.

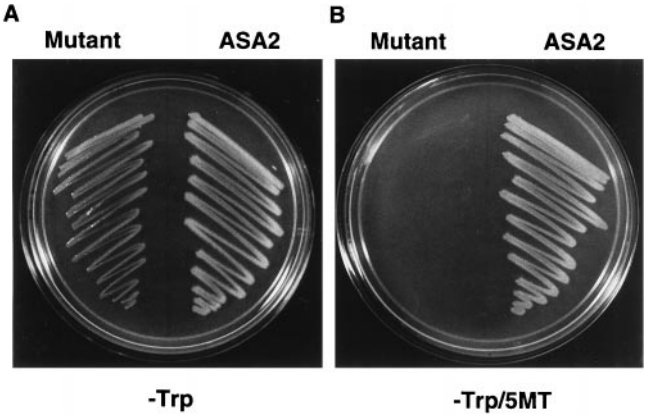

Figure 5. Complementation of *E. coli trpE5972* by the tobacco *ASA2* and a site-directed mutant. The pQE30/*ASA2* and pQE30/*ASA2* mutants were transformed into the *trpE* nonsense mutant *E. coli* strain (*trpE5972*) and plated on M9 minimal medium containing 100 μg/mL ampicillin (A) and 0.1 mM IPTG without and with 300 μM 5MT (B). The picture was taken 2 d after streaking.

feedback-sensitive AS of higher plants were changed to Phe-107 and Arg-108 (based on the tobacco *ASA2* amino acid sequence) in the tobacco *ASA2* and to Arg-108 in the *AS*α1 gene of *R. graveolens*. These changes appear to correlate with the feedback insensitivity found in the latter two enzymes.

AS Gene Expression

The tobacco *ASA2* cDNA probe detected high levels of a 2.2-kb transcript on northern blots only in 5MT^r cultured tobacco cell lines, including 5MT^r *N. sylvestris* but not in 5MT^s-cultured cell lines, leaves, roots, stems, and seeds after overnight exposure (Fig. 4).

Complementation and Inhibition Tests

The *E. coli trpE5972* nonsense mutant transformed with the tobacco *ASA2* cDNA and with the site-directed mutant ASA2 form (Phe-107-Arg-108 changed to Ser-107-Gln-108) both grew on minimal medium containing ampicillin and IPTG but no Trp (Fig. 5A). The complemented strain transformed with the site-directed mutant, however, did not grow on 300 μM 5MT containing minimal medium without Trp (Fig. 5B), whereas the growth of the strain transformed with the *ASA2* cDNA was not inhibited by 300 μM 5MT.

AS Kinetic Constants

Trp inhibition of the partially purified product of the *E. coli*-expressed tobacco *ASA2* and Arabidopsis *ASA1* genes is shown in Figure 6A. The *ASA2* gene product is still 50% active at 100 μM Trp, similar to AS in 5MT-selected tobacco cells and to purified *R. graveolens AS*α1 protein, which was 80% active at this Trp concentration (Bohlmann et al., 1996).

When AS activity was measured in extracts from wild-type and 5MT^r tobacco cell-suspension cultures the resistant cultures had a portion of the activity that is more feedback insensitive than that of the wild-type cells (Fig. 6A). The apparent K_i values for Trp are 2 and 300 μM, respectively, for AS from wild-type and 5MT-selected tobacco cells when estimated from the Trp concentration, resulting in 50% inhibition, as shown in Figure 6A. Similar values were reported by Bohlmann et al. (1996) for *R. graveolens* Trp-sensitive and -insensitive AS (2.8 and >100

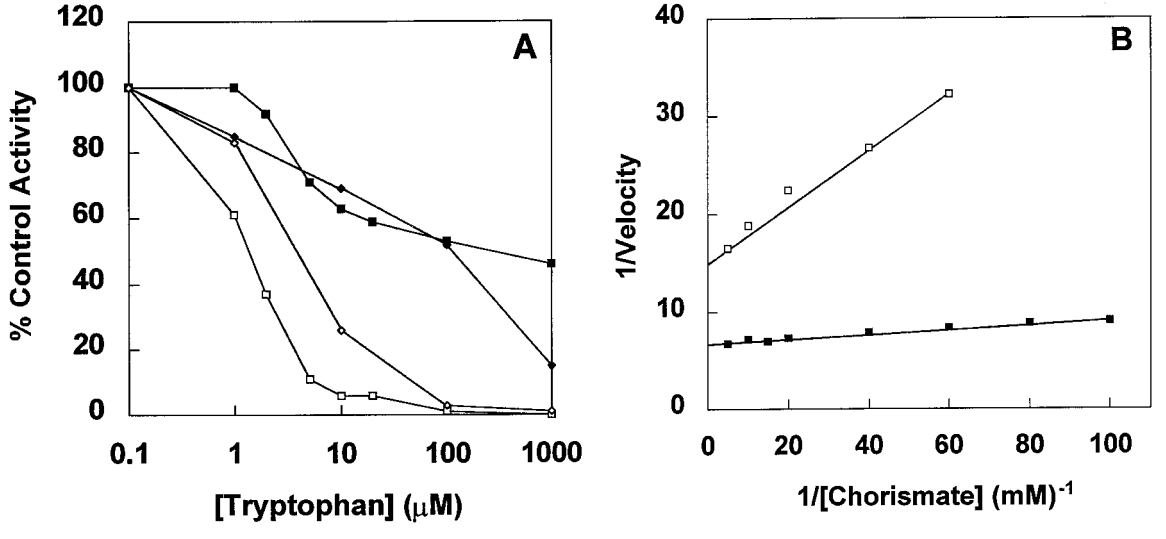

Figure 6. Kinetics of AS from wild-type (□) and 5MT-selected (■) tobacco cells and from *E. coli* transformed with the Arabidopsis *ASA1* gene (◇) or the tobacco *ASA2* gene (◆). A, Relative AS activity in the presence of Trp was measured as described in "Materials and Methods" (with 100 μM chorismate) and is expressed as a percentage of the activity with no Trp added. The specific activity with no Trp for AS from each tobacco line was 73 and 169 nmol anthranilate min^{-1} mg^{-1} protein, respectively. B. Lineweaver-Burk plot of AS activity. Velocity is expressed as nanomoles per minute per milligram of protein.

μM, respectively). AS from wild-type Arabidopsis had an apparent K_i of 3 μM, whereas AS from a 6-methyl-Trp-selected mutant had a K_i of 8 μM (Li and Last, 1996).

The apparent chorismate K_m value was lower for AS from 5MT-selected cells (6 μM) than for the wild-type cells (22 μM) when measured using 10 mM Gln as the second substrate (Fig. 6B). These values are comparable to those obtained for AS from Arabidopsis (10 and 21 μM using Gln [Li and Last, 1996]) and *R. graveolens* (17 and 29 μM using NH_4^+ [Bohlmann et al., 1996]). In all three species the chorismate K_m value (first value listed) is lower for the feedback-insensitive AS.

Comparison of *ASA2* and Wild-Type Gene Sequences

The *ASA2* fragment between +181 and +392, which contains the Phe-107-Arg-108 residues, was PCR amplified and cloned from 5MT^s and 5MT^r genomic DNAs. When the nucleotide sequence of the fragment from AB15–12-1 was aligned with those from the three wild-type *Nicotiana* sp. cell lines (Fig. 7) and compared with the *ASA2* cDNA, the genomic fragments were found to contain a 115-bp intron; therefore, the cloned fragments were 326 bp in length. The nucleotide sequences of this fragment were 100% identical in all cases except for eight nucleotides in *N. sylvestris*. This eight-nucleotide difference did not change the encoded amino acids. Amino acid sequences in this region showed 66% identity to four other plant *AS* genes from Arabidopsis and *R. graveolens*.

Promoter-Sequencing Analysis

The 99 nucleotides at the 3′ end of the promoter region and 5′ end of the *ASA2* cDNA, including the 5′ UTR, were identical (Fig. 8). The first possible TATA box (TATAAA) is located 121 bp upstream from the translation start site (ATG). BLAST analysis shows that the region from −1187 to −769 exhibits 81 to 83% nucleotide sequence identity to the promoter region of the *N. tabacum* plant-defense-related *Str246C* gene (Froissard et al., 1994).

Chimeric GUS Gene Expression

When different deleted fragments of the ASA2 promoter region were tested, the transient GUS expression controlled by the promoter region denoted 606 was stronger in both suspension-cultured cells and in leaves than that driven by the CaMV 35S promoter (Fig. 9). The 1356 and 2252 regions still showed high GUS activity in suspension-cultured cells but very low activity in leaves. The 1356 region showed approximately 5 times higher GUS expression than that driven by the CaMV 35S promoter in 5MT^r suspension-cultured cells.

To determine whether the tissue culture-specific expression controlled by the ASA2 promoter was caused by transcription factor(s) in the 5MT^r cell lines, we performed separate experiments using suspension-cultured cells from 5MT^s *N. tabacum* and from 5MT^s and 5MT^r *N. sylvestris*. The transient GUS activities in the *N. tabacum* 5MT^r suspension-cultured cells (AB15–12-1) controlled by 35S, 606, 1356, and

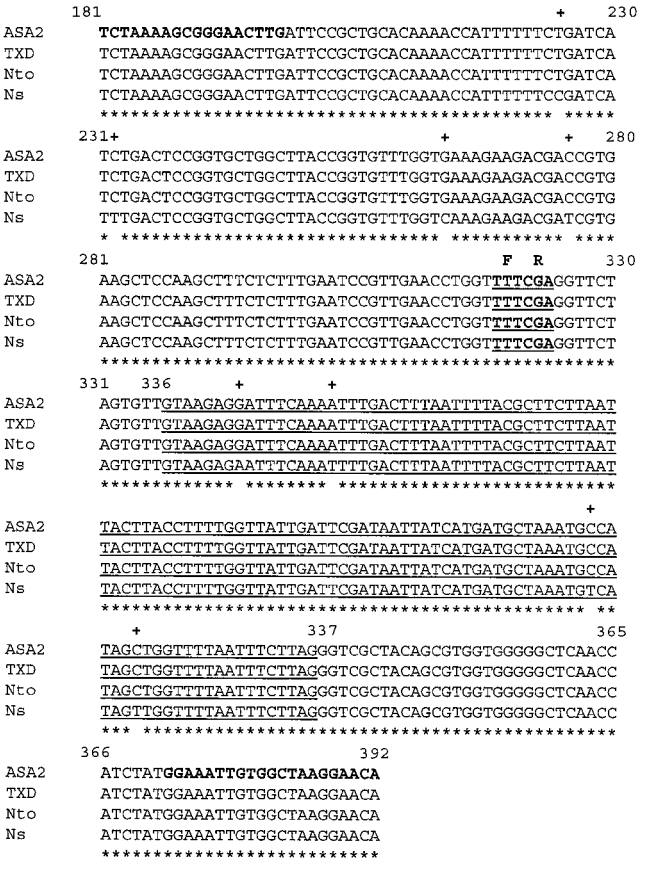

Figure 7. Nucleotide sequence alignment of *ASA2* genomic DNA fragments. The 326-bp *ASA2* genomic DNA fragments, corresponding to the *ASA2* cDNA sequence from +181 to +392, were amplified from the genomic DNAs of tobacco leaves (ASA2) harvested from AB15–12-1 shoot cultures and three wild-type (5MT^s) *Nicotiana* sp. cultured cell lines, *N. tabacum* (TXD), *N. tomentosiformis* (Nto), and *N. sylvestris* (Ns) using primers 4 (sense) and 10 (antisense), which are shown in bold. These genomic DNA fragments contain a 115-bp intron, which is underlined. Eight nucleotides, indicated by (+) above the sequence, are different in the *N. sylvestris* genomic fragment compared with those in *N. tabacum* and *N. tomentosiformis*. The nucleotide sequences corresponding to Phe-107-Arg-108 are indicated in boldface and underlined with the one-letter amino acid codon abbreviations F and R above the sequence.

2252 promoters were approximately 1.8-, 7.4-, 3.7-, and 3.6-fold that of the 5MT^s tobacco suspension-cultured cells, respectively. In the case of *N. sylvestris*, 3.4-, 10.6-, 6.4-, and 9.1-fold GUS activities were detected in the 5MT^r suspension-cultured cells in comparison with the 5MT^s suspension-cultured cells, respectively.

That the ASA2 promoter shows tissue-specific expression was also shown by stable transformation experiments with tobacco using *Agrobacterium tumefaciens* (data not shown).

DISCUSSION

We have cloned the tobacco *ASA2* cDNA encoding the α-subunit of a feedback-insensitive AS and the promoter region. Southern hybridization analysis shows that more

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CTAGTTATGGATGAGGACAGGTTCATTGAAGCTTCAAATCTATTCGATAGTGGGACCTACGTCTCAAATCCCGAAAAAAC  80
TCGCGAAATCCGAACACCCGTTCCGCTACGAGTTCAACCATACAAAAATTATCCAATTCTGATGTCAACTCGACCCTCAA  160
ATCTTCAATTAAAGTCTTTGAAGACTTCTATCATTTTCAACTCAATCTTTATCCCATTTGAACTAAACACTATTTCCATA  240
AAACCTTATTGATACGTATAAATAATACTCTTACACCCAAGAATTATACTCTTAATCACCCATCATTACCCAAACTCGGA  320
ATTGAAGATTAAAACCTTACCTCTTTGATGAAGAACTTGAGGGATTTTTTTGTTGGATTTCAAGGCTTGGACAAGAATTT  400
GATGAGCAAGACACTTTATCTACTTCCTCTCTCTAGAACACTCTCACTTCTCTCTAAAATCATCAGATAGTTGCCCCAAA  480
ACCTATTTATCAAAATAGAGTCGGGTAATGAAAATAGGTAAATGGACCCTCCAAACTCAGGTATGCGATTGCACAATGGA  560
TATACGGGTCGCACAATGGACCACCAAATCGATGCCGAAAACTGGGTTGCGCTGGACAGGTCTGCGACCCATTTTACGGT  640
CGCACAATGTGCTACGAAGAGGAATTCACATAGATTTAGGAAGGGCCTGTTGTATTTGTGTACAAGCTAAAGTTTTTTGA  720
AAAACAAATACCTTTGGTCACTTTCATTGTCAAATAGGTTTTTCCTTCGTATACCTTACTTACATCACATAGTGATTATG  800
CGATCGCACAATTTACCGCATAATCGTATTTTTCCAGCTTTTGGTAATTTAATCATAACTTTTTTTATGAATATCCAAAT  880
GACGAACTGTTTGAAGCGTTAGAAACTAGACTCAAAGATCTTTCATTTTATAGGCAATACGGCACATAATATTTTGTATC  960
ATGAGAGTTATTCTCATTTGAAGTTAGGTCTTGTGTGAACTCACTTGAAACTTTAGTCTTATGAAATTTCCAACTTCTAC  1040
ATCCGATTCCGAAACCTATCGAATCAAGTCCGATTGACCTCAAATTTTGCATACAAGCCATAAATGACATAACAGAGCTA  1120
TAAAATTTTTCGAAACGGGATTCCGGCTCCGATATCAAAAAGTCAACCCTGTGGTCAAACTTGGAAATCTTTAGCCTTTA  1200
AATTACTAGTTTCCGTTAAATGGTCATAACTTGAGTTATGGACCTCCAAATTAAATTCCGGGCATACGCCCAAGTCCCAT  1280
ATCACGATACGAACCTATAGGAACTTTCAAAATATTGATCCGGATCCGTTTGCTCAAAATGTTGATCAAAGTCAACTCAG  1360
TTGAGTTTTAAGGCTCTAGTTCACATTTTAATCCATTTTCACCTAAAAACTTTCCGGAAAATTTTACGGATTTCGCACGC  1440
AAGTCGATGAATGACTTTTGGAGGTCTTAGAACACGTAATTAATTATTAAATTTAAAGATGACATTTTGGATAATCACCC  1520
AAGTAGTACAAATTTTTTATGCGGTGATTATATTTGCCAATCCATCAAGCCAAACATGTCGTAATTAGTCATAAATTAAG  1600
TTATACAGGAAGAATAATACGAGAAATATAATACCTAAATTAATAAATACTACTATAAAATTATAATATTGATATTGTGG  1680
TTGTATTGCCCATTTCATTAGAAAGGATATATGATGTATAATATAAAATTTTACAATGTTATTCTTGTTTTTAAAGTTAA  1760
TAAAAATTTAAAATATGAATTTAAGGTTATTCTTGTTTATAGATTCTTTATATCATAAAGCTAATCCTCGTATAAATTAT  1840
TTCATATTCGACTCATATAAACTAATACTGAAATTACTATATAAGATTATATACCGGTATATATTGGAAACGAGACATCA  1920
GCCAAATGTGTCCAAAAATAATAAATATCAAATTTTATATCAGGATTATTTTTTTTGATTATGTTAACAAAGTTAAAAGT  2000
ATCAGACTATAAATACTGTAGATAAGATCAGCCATTATTAGAGATAATACTCTCACTACCTATATTGAAAGTGAAGTAGA  2080
CATTTTCTGAGGTGGAATATTTAAAACGTTTTCAGACATTTAAAACCTGGAATGCGGAGGCAAAGTAGTGTAGTACTTAC  2160
TAGTAGTATAAATAAGTGATCCCATTTTCAAAGTCACCGTCAAAAATCCCCATTTCACCGTTTCCTCGTTTCTCCTCCTC  2240
ACTAATTTTGTCTCTTTCTCTTGGTTTGCTATTGTGCTCTTGTAGGAATGCAGTCG                          2287
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Figure 8. The nucleotide sequence of the ASA2 promoter region. The first possible TATA box (TATAAA), translation start codon (ATG), is indicated in boldface. The 98 bp at the 3′ end of the ASA2 promoter region, which is identical to the nucleotide sequence at 5′ end of the *ASA2* cDNA, is underlined.

than one closely related sequence is present in the tobacco genome (Fig. 2). Like all plant AS genes described to date, the tobacco *ASA2* has a putative transit peptide sequence; therefore, the enzyme is apparently localized in the plastids.

Our results support the conclusion that *ASA2* is a wild-type gene encoding the α-subunit of a feedback-insensitive AS in tobacco. These results include the feedback-insensitive *ASA2* gene expression in the *trpE* mutant *E. coli* strain (Fig. 5), enzyme kinetic analysis (Fig. 6), the nucleotide sequence of wild-type *ASA2* genomic fragments (Fig. 7), and high-level expression in 5MT^r suspension-cultured cells (Fig. 4).

Several regions of the AS amino acid sequence have been shown to affect feedback inhibition (Bohlmann et al., 1996; Kreps et al., 1996; Li and Last, 1996). In tobacco *ASA2* from both wild-type and 5MT^r cell lines, the two amino acids Phe-107 and Arg-108, found in the same region as in *ASα*1 (Bohlmann et al., 1996), were different from those found in feedback-sensitive AS, Ser-107 and Gln-108. When we changed the *ASA2* Phe-107 and Arg-108 residues to Ser-107 and Gln-108 by site-directed mutagenesis, the gene product was still active in *E. coli*, but this complemented *E. coli* strain could not grow on 300 μM 5MT containing minimal medium without Trp, whereas the original ASA2 complemented strain could. In addition, the *E. coli*-expressed *ASA2* gene product was feedback insensitive like the AS found in 5MT^r tobacco cell extracts. These results indicate that the Phe-107 and Arg-108 residues are important in the control of feedback inhibition. Further mutagenesis and kinetic and binding studies will be necessary to determine the effect of other amino acid changes in this region.

The apparent Trp K_i values for feedback-sensitive AS from tobacco, *R. graveolens*, and Arabidopsis are similar, as one might expect considering the high degree of identity in the regions known to affect feedback inhibition of bacterial AS, which are also assumed to be important in plant AS (Caliguri and Bauerle, 1991; Bohlmann et al., 1995). The apparent Trp K_i values for the tobacco and *R. graveolens* feedback-insensitive AS enzymes are similar to each other and are much higher than the K_i value of the mutant Arabidopsis feedback-insensitive AS. This pattern of inhibition constants correlates with the sequence homologies observed and supports the hypothesis that the substitution of Arg-163 in *R. graveolens* (corresponding to Arg-108 of tobacco) and Phe-107-Arg-108 in tobacco AS are related to higher feedback insensitivity than that found in the Arabidopsis mutant AS when Asp-341 is changed to Asn.

That the apparent K_m values for chorismate for tobacco AS are similar to the values obtained for Arabidopsis and *R. graveolens* AS is also not surprising considering the high degree of similarity, especially in the region known to be involved in catalysis for bacterial AS (Caliguri and Bauerle, 1991; Bohlmann et al., 1995). In all three species the apparent K_m for chorismate of the feedback-insensitive AS is lower than the K_m for feedback-sensitive AS. This suggests a similar mechanism of inhibition and feedback insensitivity. This would be expected for tobacco and *R. graveolens*, since a similar sequence difference appears to be involved, but the Arabidopsis mutant contains a different structural change that produces an enzyme that is still appreciably inhibited by Trp.

The results presented here also indicate that the tobacco ASA2 promoter regulates tissue-specific gene expression.

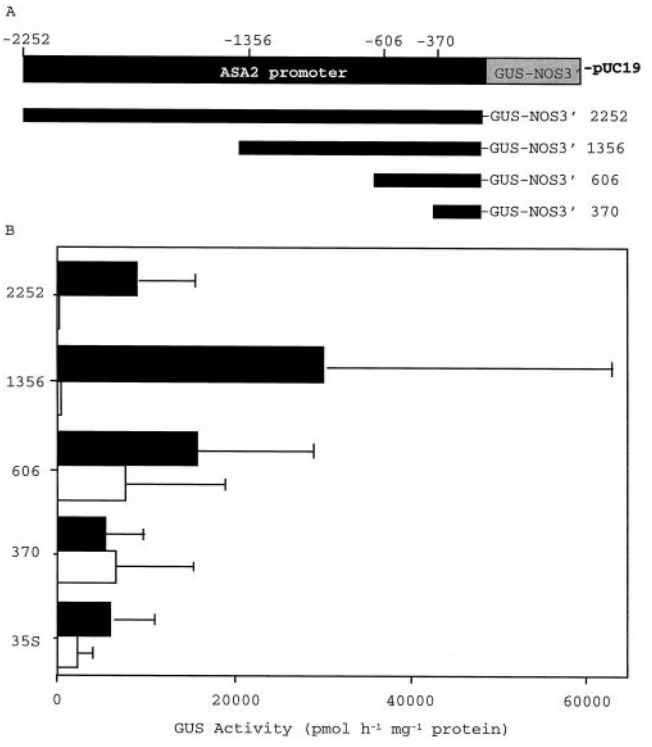

Figure 9. Chimeric *ASA2* promoter-GUS constructs and transient GUS assays with tobacco leaves and suspension-cultured cells. Four deleted promoter fragments were fused to the GUS reporter gene with NOS terminator in pUC19 and designated 2252, 1356, 606, and 370. These chimeric constructs and the CaMV 35S-GUS construct as a control were bombarded into 2-d-old AB15–12-1 cultured cells (black bars) and leaves (white bars) harvested from 3-week-old shoot cultures. GUS transient expression was measured using the MUG assay 3 d after bombardment. The average values after subtracting the background from four separate experiments with two replicates are presented. GUS-specific activity was determined as picomoles of 4-methylumbelliferone per hour per milligram of protein.

This conclusion is supported by measurements of gene expression at the mRNA level (Fig. 4) and by the deletion-analysis studies of the ASA2 promoter region (Fig. 9). The high level of *ASA2* gene expression was detected at the mRNA level only in $5MT^r$ suspension-cultured cells, which is similar to the previous results found at the enzyme activity level (Brotherton et al., 1986). In addition, the transient GUS expression was higher in the $5MT^r$ than in the $5MT^s$ suspension-cultured cells and very low-level expression was found in leaves. This tissue-specific expression was controlled by the promoter region between −2252 and −607. These results suggest that there could be changes in the amount or type of regulatory protein(s) in the $5MT^r$ suspension-cultured cells that up-regulate the *ASA2* gene expression. Changes in a *cis*-acting element(s) in the promoter region can probably be excluded, since we have found only a few nucleotide differences in the ASA2 promoter region isolated from three wild-type tobacco cell lines in comparison with ASA2. We also found similar transient expression patterns with these promoters compared with that found with the ASA2 promoter from AB15–12-1 (data not shown).

In Arabidopsis and *R. graveolens* the *ASA1* and *ASα1* gene expression is inducible, whereas the *ASA2* and *ASα2* genes are expressed constitutively. Even though we have denoted our clone *ASA2*, since the highest amino acid identity was with *R. graveolens ASα2* (72%) and since we have isolated a truncated *ASA1* cDNA similar to the Arabidopsis *ASA1* (data not shown), the tobacco *ASA2* is also very similar to *R. graveolens ASα1* (70% identity). So far, we have not been able to induce leaf *ASA2* mRNA synthesis by treatment with auxin or salicylic acid (within 8 h) or by wounding (within 48 h). To help understand in vivo ASA2 gene expression in plants and the effect of environmental conditions on gene expression, we have recently produced tobacco plants transformed with the GUS reporter gene driven by the 2252-bp ASA2 promoter. The feedback-insensitive *R. graveolens ASα1* appears to be involved in the pathway synthesizing anthranilate-derivative alkaloids (Bohlmann et al., 1996). However, we are not aware of any tobacco anthranilate or indole-derived phytoalexins or alkaloids that have been identified; therefore, the function of the *ASA2* gene in tobacco has yet to be determined.

In this paper we have reported the cloning of a wild-type tobacco *ASA2* gene encoding the α-subunit of a feedback-insensitive AS. Feedback insensitivity in the $5MT^r$ suspension-cultured cells was correlated with the high level of the *ASA2* gene expression and with the tissue-specific expression of this gene controlled by defined promoter regions. The role of the feedback-insensitive AS in tobacco is still unknown and further studies are needed to understand the regulation and function of this enzyme in plants.

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