Tissue-Specific Expression of the β-Subunit of Tryptophan Synthase in *Camptotheca acuminata*, an Indole Alkaloid-Producing Plant

Hua Lu and Thomas D. McKnight*

Department of Biology, Texas A&M University, College Station, Texas 77843

Camptothecin is an anticancer drug produced by the monoterpene indole alkaloid pathway in *Camptotheca acuminata*.* As part of an investigation of the camptothecin biosynthetic pathway, we have cloned and characterized a gene from *C. acuminata* encoding the β-subunit of tryptophan (Trp) synthase (TSB). In *C. acuminata* TSB provides Trp for both protein synthesis and indole alkaloid production and therefore represents a junction between primary and secondary metabolism. TSB mRNA and protein were detected in all *C. acuminata* organs examined, and their abundance paralleled that of camptothecin. Within each shoot organ, TSB was most abundant in vascular tissues. Within the root, however, TSB expression was most abundant in the outer cortex. TSB has been localized to chloroplasts in Arabidopsis, but there was little expression of TSB in *C. acuminata* tissues where the predominant plastids were photosynthetically competent chloroplasts. Expression of the promoter from the *C. acuminata* TSB gene in transgenic tobacco plants paralleled expression of the native gene in *C. acuminata* in all organs except roots. TSB is also highly expressed in *C. acuminata* during early seedling development at a stage corresponding to peak accumulation of camptothecin, consistent with the idea that TSB biosynthesis and the secondary indole alkaloid pathway are coordinately regulated.

The Trp biosynthetic pathway in plants (for review, see Radwanski and Last, 1995) has several important roles in addition to providing Trp for protein biosynthesis. This pathway also supplies precursors for the biosynthesis of the phytohormone auxin and indole alkaloids, including the anticancer drugs vinblastine, vincristine, and camptothecin. Camptothecin is a monoterpene indole alkaloid produced by *Camptotheca acuminata*, a tree native to China. Camptothecin inhibits DNA topoisomerase I (Kjeldsen et al., 1992) and is therefore preferentially toxic to rapidly dividing cells. The anticancer properties of camptothecin were discovered in the 1960s (Wall et al., 1966), but severe side effects, mostly stemming from its near insolubility in aqueous systems, stopped clinical trials in the 1970s. Currently, two semisynthetic derivatives that are more soluble and less toxic are used in the treatment of a number of cancers (for review, see Dancey and Eisenhauer, 1996).

Because Trp biosynthesis is required for both primary and secondary metabolism in *C. acuminata*, we were interested in determining how this pathway is expressed and regulated. Trp biosynthesis begins with the conversion of chorismate to anthranilate by anthranilate synthase. After production of the intermediates 5-phosphoribosylanthranilate and indole glycerol phosphate, TSA produces indole, which is then condensed with Ser by TSB to form the final product. The entire Trp pathway has been localized to the plastid (Zhao and Last, 1995), but all of the enzymes are encoded by nuclear genes. In both maize (Wright et al., 1992) and Arabidopsis (Last et al., 1991), TSB is encoded by two distinct genes. The two Arabidopsis TSB genes are differentially expressed. TSB1 mRNA is most abundant in rosette leaves and less abundant in inflorescences, flower buds, and roots. TSB2 appears to be expressed at a consistent, low level throughout the plant (Pruitt and Last, 1993).

In maize there are also apparently two genes encoding TSA. One of these genes, designated Bx1, is dedicated to producing indole for use in the biosynthesis of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one, a secondary product that provides an effective defense against insect pests and fungal pathogens (Frey et al., 1997). The second TSA gene is presumably associated with primary metabolism and produces indole for conversion to Trp by TSB.

TSB provides the indole moiety for monoterpene indole alkaloid biosynthesis. Trp is decarboxylated by TDC to produce tryptamine. Tryptamine is then conjugated to the terpenoid secoliganin, to form the key intermediate strictosidine. Strictosidine is a precursor to more than 1800 alkaloids, including camptothecin (Kutchan, 1995). The *C. acuminata* genome encodes two TDC genes that are differentially expressed. TDC1 expression is correlated with the sites and times of camptothecin accumulation. TDC2 expression is very low in all of the tissues examined but can be induced by mimicking a pathogen attack with a fungal elicitor or methyl jasmonate (López-Meyer and Nessler, 1997).

We used antibodies and nucleic acid probes to investigate the expression of TSB in *C. acuminata*. The protein is expressed at a high level in the vascular tissues of young saplings and during a very early seedling stage that immediately precedes a peak of camptothecin accumulation, suggesting that TSB biosynthesis and the indole alkaloid pathway are coordinately regulated.

Abbreviations: RPA, ribonuclease protection assay; TDC, Trp decarboxylase; TSA, Trp synthase α-subunit; TSB, Trp synthase β-subunit.

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* Corresponding author; e-mail mcknight@bio.tamu.edu; fax 1–409–845–2891
MATERIALS AND METHODS

Plant Materials

Camptotheca acuminata seeds were surface-sterilized with 10% Triton X-100 (5 min), 70% ethanol (1 min), and 1% bleach (3 min) followed by thorough rinsing with water. Seeds were then germinated on a Murashige and Skoog medium (Murashige and Skoog, 1962) in sterile boxes (Magenta Corp., Chicago, IL) and grown at 25°C under a 16-h light/8-h dark cycle. Seedlings were collected on different days after imbibition and frozen in liquid N2 for further analysis. One-year-old C. acuminata trees were grown under natural light in a greenhouse.

Cloning of C. acuminata TSB cDNA and Gene

A DNA fragment from the Arabidopsis TSB1 cDNA (a kind gift from Dr. Robert Last) was radiolabeled with a random primer labeling kit (Amersham). This probe was used to screen a C. acuminata cDNA library constructed from 7-d-old seedlings (Burnett et al., 1993). Seventeen cDNA clones were isolated from 3 × 10⁵ phage particles of the primary cDNA library. Restriction mapping and partial sequencing analysis indicated that all of the 17 clones were derived from the same gene, with some of them containing truncated inserts. One of the longest clones was completely sequenced. A 515-bp EcoRI/BglII fragment from the 5’ end of the C. acuminata TSB cDNA was radiolabeled and used to screen a C. acuminata genomic library (Burnett et al., 1993). Six plaques were isolated from 5 × 10⁵ recombinants (approximately 4 genome equivalents) and appeared to be identical by DNA restriction analysis. One of these plaques was purified and the 15-kb insert was subcloned into pUC18. The C. acuminata TSB gene was designated CaTSB1.

Nucleotide Sequencing and Analysis

Nucleotide sequences were determined by the dye-terminator cycle sequencing method (ABI Prism Dye Terminator cycle sequencing core kit, PE Applied Biosystems, Foster City, CA) with an automated sequencing system. The cDNA and 9.4 kb of the genomic clone were sequenced on both strands. DNA sequence assembly and mapping analysis were performed with Sequencher (version 3.0, Gene Code Corp., Ann Arbor, MI). The Geneworks program (version 2.3, Intelligenetics, Mountain View, CA) was used for amino acid comparison. The sequences reported here appear in the nucleotide sequence databases under the accession nos. AF042320 and AF042321 for the cDNA and genomic sequences, respectively.

Nucleic Acid Isolation and Analysis

DNA was isolated from leaves of a 1-year-old C. acuminata tree, using a method described by Nagao et al. (1981). DNA (10 µg/lane) was digested with restriction enzymes, separated in a 0.8% agarose gel by electrophoresis, and then transferred to a nylon filter (MSI, Westboro, MA) according to the manufacturer’s instruction. Hybridization was performed overnight at 55°C in hybridization solution (5 × Denhart’s solution, 5 × SSC, 0.1% SDS, 5 mm sodium PPI, and 50 µg ml⁻¹ denatured salmon testes DNA). A 933-bp SacI/HindIII fragment from the TSB cDNA was used to probe the filter. The filter was washed with 5 × SSC and 0.1% SDS for 20 min once and 2 × SSC and 0.1% SDS for 30 min three times at 55°C. TSB mRNA was detected by ribonuclease protection assays. A 771-bp BglII/HindIII fragment was cloned in pBluescript SK+ and used to generate an antisense probe. The antisense RNA probe was synthesized by using T3 RNA polymerase (MAXScript in vitro transcription kit, Ambion, Austin, TX) after linearization with XhoI. The protected bands were detected with a Direct Protect kit (Ambion) and separated on a 5% polyacrylamide gel. A 250-bp antisense probe from a C. acuminata rRNA clone (López-Meyer and Nessler, 1997) was used to normalize the variations of total RNA for each sample. Autoradiography was done by exposing the gels to x-ray film at ~80°C. Relative amounts of mRNA were quantified on phosphor imaging screens with a Fujix BAS 2000 Bio-Imaging Analyzer (Fuji, Tokyo).

The HindIII site used in several constructions was created in the cDNA by splicing together exons 3 and 4 and is not present in the genomic sequence.

Expression and Purification of TSB-His Tag Protein and Antibody Production

A 933-bp SacI/HindIII fragment from the TSB cDNA was subcloned into the expression vector pET23a+(+) (Novagen, Madison, WI). After insertion of the cDNA, the NcoI site in the vector was cut and end-filled with the Klenow fragment of DNA polymerase I to place the His tag of the vector in-frame with TSB. A 33-bp sequence at the 5’ end of the vector gave a 12-amino acid peptide fused to the TSB protein. A monoclonal antibody against this short peptide, the T7 tag antibody (Novagen), was used to confirm expression. The construct was transferred to the BL21(DE3)pLysS Escherichia coli strain. Expression was induced by adding 0.4 mm isopropyl-β-D-thiogalactoside (Sigma) to bacterial cultures at an optical density of 0.6, which were allowed to grow for an additional 5 h. A His Bind-resin (Novagen) column was used to purify the expressed protein, according to the protocol provided by the manufacturer. Protein samples from the elution step of the column were purified further by preparative SDS-PAGE, and a single band of TSB protein was obtained. The purified TSB protein was emulsified with the RIBI adjuvant system (RIBI ImmunoChem Research, Hamilton, MT) and injected into rabbits, 100 µg each time, at 0, 4, 6, and 8 weeks.

Protein Blotting and Analysis

Total protein was extracted from C. acuminata tissues with lysis buffer (0.125 M Tris, pH 6.8, 4% SDS, 20% glycerol, 0.002% bromphenol blue, and 5% β-mercaptoethanol) and quantified by the Lowry assay (Lowry et al., 1951). Twenty micrograms of protein sample per lane was resolved on a 7.5% SDS-PAGE gel and electroblotted onto a...
The subclone was digested using HindIII and was ligated into pBluescript SK+ at the restriction sites. The constructs were then transferred into the pNco-GUS vector in liquid N₂. The recombinant plasmids were propagated in the E. coli strain LB4404 and transferred to the Agrobacterium tumefaciens C58. The promoter::GUS constructs were then transformed into tobacco (Horsch et al., 1985).

**RESULTS**

Isolation and Analysis of the *C. acuminata* TSB Gene

The Trp biosynthetic pathway is highly conserved in microorganisms and plants (Crawford, 1989; Radwanski and Last, 1995). Sequence comparisons show that genes encoding enzymes in the pathway have a high degree of similarity among different organisms. We used the heterologous TSB1 cDNA probe from Arabidopsis to screen a *C. acuminata* seedling cDNA library (Burnett et al., 1993), and 17 positive clones were isolated. Partial sequencing revealed that all 17 clones were derived from identical mRNAs. One of the longest clones was completely sequenced and found to contain an apparent full-length cDNA.

The *C. acuminata* TSB cDNA contained 1707 nucleotides with an open reading frame encoding a protein of 466 amino acids. The 5'-untranslated region contained 60 nucleotides and the 3'-untranslated region contained 270 nucleotides. No consensus polyadenylation signal was found in the 3'-untranslated region. A database search with the deduced TSB amino acid sequence revealed significant similarity to previously characterized TSB proteins from both prokaryotes and plants. An alignment of deduced amino acid sequence of *C. acuminata* TSB to other plant TSB proteins is shown in Figure 1. Similarity between *C. acuminata* TSB and either of the two Arabidopsis TSB proteins was 80%, whereas similarity between *C. acuminata* TSB and the two maize TSB proteins, TSB1 and TSB2, was 74% and 78%, respectively. Although the overall similarity among plant TSB proteins is very high, the first 68 amino acids from the N terminus of the predicted *C. acuminata* TSB are not conserved. This domain has a high Ser and Thr content, a feature also found in the amino-terminal domain of TSB proteins from Arabidopsis and maize and a feature that is characteristic of plastid transit peptides. Subcellular fractions and immunoblot analysis confirmed that Arabidopsis TSB proteins are localized to plastids (Zhao and Last, 1995).

The corresponding gene, designated *CaTSB1*, was then identified by screening a *C. acuminata* genomic library (Burnett et al., 1993) using a 515-bp fragment (EcoRI/BglII) from the 5' end of the *C. acuminata* TSB cDNA as a probe. The gene sequence from the initial ATG codon to the termination codon is 646 bp. Approximately 2500 bp of DNA 5' to the start codon was also sequenced. The coding region was divided among five exons. The four introns were separated from the exons by typical GT/AG dinucleotide boundaries. The nucleotide sequence of the *CaTSB1* exons, including 5'- and 3'-untranslated regions, was identical to that of the cDNA.

Southern blotting was performed to determine the copy number of TSB genes in the *C. acuminata* genome. A nonradioactive method was used to detect the TSB probe.
A genomic DNA gel blot was probed with a radiolabeled 933-bp restriction fragment from the coding region of the cDNA and then washed under low stringency conditions (Fig. 2). All of the hybridizing bands, except for the smallest XmnI band, were consistent with the restriction pattern of the CaTSB1. This XmnI band could be from an uncloned allele of CaTSB1 or from a second TSB gene with a restriction pattern nearly identical to CaTSB1. Without the complete sequence of the genome, it is impossible to rule out the presence of additional TSB genes that were too divergent to be detected by Southern analysis. However, the high degree of conservation of TSB genes across species (Fig. 1) made the latter possibility unlikely.

Figure 1. Comparison of the deduced amino acid sequences of TSB genes from C. acuminata, Arabidopsis, and maize. Line 1 represents the consensus sequence derived from the individual genes. Dots indicate positions where there is no consensus (mostly in the transit peptide), and dashes indicate regions that are deleted in two or more proteins. Line 2 represents TSB derived from the CaTSB1; lines 3 and 4 represent TSB derived from TSB1 and TSB2, respectively, from Arabidopsis; and lines 5 and 6 represent TSB derived from the TBS1 and TSB2, respectively, from maize. In the individual sequences, dots indicate agreement with the consensus sequence, and dashes indicate absence of the corresponding amino acid.

Figure 2. Genomic Southern analysis of TSB sequences. A, Restriction map of CaTSB1. B, Autoradiograph of a genomic Southern blot probed with a 933-bp SacI-HindIII fragment from the center of the coding region of the TSB cDNA. This fragment contains part of exon 1 and all of exons 2 and 3. The HindIII site was created in the cDNA by splicing together exons 3 and 4 and is not present in the genomic sequence. Ten micrograms of C. acuminata DNA was digested with the indicated enzymes, separated on a 0.8% agarose gel, and transferred to a nylon membrane. The membrane was probed with [α-32P]dCTP-labeled TSB cDNA at 55°C. The blot was washed with 2× SSC and 0.1% SDS three times for 30 min at 55°C. All of the hybridizing bands are consistent with the restriction map of CaTSB1, except for a very faint XmnI band at 1.6 kb.
Expression of TSB Protein in *C. acuminata*

To examine TSB protein levels, we expressed the cDNA in *E. coli* and raised antibodies against the recombinant protein in rabbits. Immunoblots were performed to analyze the expression of TSB in different parts of 1-year-old *C. acuminata* trees (Fig. 3). TSB protein was detected in all organs of the trees, with higher levels in the apex, bark, young leaf, and young stem and lower levels in old leaf, auxiliary bud, and root. This pattern of expression paralleled the accumulation of camptothecin in these organs (López-Meyer et al., 1994).

Further localization of TSB protein in *C. acuminata* plants was determined by tissue printing. Cross-sections of fresh tissue were printed on a nylon membrane. The membrane was then treated as a protein blot using anti-TSB serum as the first antibody and alkaline phosphatase-conjugated goat anti-rabbit IgG as the secondary antibody. Figure 4 shows the deposition of TSB protein in the *C. acuminata* stem, petiole, and root and the anatomical structure of each tissue. In the stems of a 1-year-old tree, TSB was abundant in vascular tissues, especially the cambium, primary xylem tissue, and phloem. No TSB was evident in the epidermis, cortex, or pith. This protein localization pattern was confirmed in the stem section of 30-d-old seedlings, in which the vascular tissue was strongly stained. A similar TSB localization pattern was found in petioles. In roots, however, TSB was found in the subepidermal cortex and a lesser amount was found in the central vascular tissue. No TSB protein was seen in the inner cortex. Despite the need for Trp in all metabolically active cells, expression of TSB was largely confined to vascular tissues in the shoots and the outer cortex in the roots of *C. acuminata*.

Expression of TSB during *C. acuminata* Seedling Development

Results from western blotting (Fig. 3) indicated a correspondence between TSB expression and organs that accumulate high levels of camptothecin in 1-year-old saplings (López-Meyer et al., 1994). Young seedlings have a peak of camptothecin production at 10 to 12 d postimbibition, which is preceded by induction of the Trp decarboxylase gene *TDC1* (López-Meyer and Nessler, 1997). To further investigate the correlation between alkaloid production and TSB expression, we examined young seedlings.

RNase protection assays showed that Ca*TSB1* mRNA was transiently induced during seedling growth, with a peak in 6-d-old seedlings (Fig. 5A). This same batch of seedlings was analyzed by López-Meyer and Nessler (1997) for camptothecin production and TDC expression. Expression of Ca*TSB1* mRNA peaked earlier than *TDC1* and camptothecin accumulation (d 10 and 12 postimbibition, respectively). The accumulation of Ca*TSB1* mRNA was followed by an increase in TSB protein, as detected by western blotting (Fig. 5B). TSB protein increased rapidly after seedling imbibition and reached a maximum in 7-d-old seedlings. The protein level then began to decrease and reached a steady state 9 d after imbibition.

Because TSB must provide Trp for both protein and alkaloid synthesis, we examined total protein levels to determine whether TSB expression was also correlated with increased protein synthesis. A rapid decrease in total protein was observed within the first 5 d, followed by a slower decline over the next 6 d (Fig. 5C). This decline presumably represented the degradation of storage proteins, which should increase the pool of amino acids available for protein synthesis. The amount of Trp contained in the storage proteins of *C. acuminata* is not known, and a low level may require de novo Trp synthesis during germination. However, there appeared to be no massive burst of protein synthesis at the time of maximal TSB expression. The peak of camptothecin accumulation occurred in 12-d-old seedlings (López-Meyer and Nessler, 1997), and the peak of TSB protein in seedlings less than 12 d old may have reflected the requirement of Trp as a precursor for camptothecin biosynthesis.

Expression of Ca*TSB1::GUS* in Transgenic Tobacco

It was possible that the pattern of TSB expression determined by tissue printing was biased by the cellular structure of the tissues. For instance, proteins from cells with more water or weaker cell surfaces could have deposited more protein on the filter. To examine TSB localization in

![Figure 3](https://example.com/figure3.png)
another system, we fused the promoter region of CaTSB1 to the reporter GUS gene and transformed this chimeric gene into tobacco. As indicated in Figure 6, the longest promoter region tested (893 bp) gave the strongest expression when analyzed in 6-d-old seedlings. Decreased GUS expression was found as the promoter length decreased from 893 to 220 bp, suggesting that multiple cis elements located within this region quantitatively affected the expression of the CaTSB1 promoter.

We used histochemical GUS staining to localize the expression of the CaTSB1 promoter in the tobacco tissues. A typical GUS-staining pattern from transgenic tobacco plants carrying the 893-bp CaTSB1 promoter appears in Figure 7. Expression was particularly strong in young stems, mainly in vascular tissue (Fig. 7A). No staining was seen in the pith, epidermis, cortex, or trichomes of the stem. In older stems there was no expression in vascular tissue, except at the nodes where new lateral buds were forming (Fig. 7B). A weak but reproducible GUS stain was observed in the vasculature of petioles (Fig. 7C). In 8-d-old tobacco seedlings, GUS expression was detected in the hypocotyl and to a lesser extent in the lower part of the cotyledons (Fig. 7D). Expression in all of these organs rapidly faded as the seedlings grew. In the leaves of mature tobacco plants, the veins, but not the mesophyll cells, were stained blue (not shown). GUS staining in the shoot correlated well with the tissue-printing results from C. acuminata, suggesting that localization of TSB in vascular tissue was due to signals within the 893-bp promoter region. Because tobacco is not known to produce indole alkaloids, this pattern of expression was not due to a demand for Trp by secondary product pathways in the vascular tissue. The same pattern of tissue-specific expression was seen for all CaTSB1 promoters down to 220 bp (not shown), indicating that the sequences for vascular expression lay close to the beginning of the gene.

Despite the correlation between the expression pattern for TSB in C. acuminata shoots and CaTSB1::GUS in tobacco shoots, expression in tobacco roots was variable. Only 4 of the 10 transgenic lines expressed GUS in the roots of mature plants, mostly in apical and lateral root meristems and regions around the lateral root-branching sites (not shown). A similar pattern of weak and variable GUS expression was reported for the Arabidopsis TSB1 promoter in the roots of transgenic Arabidopsis (Pruitt and Last, 1993). In contrast, tissue printing showed abundant expression of TSB in the root epidermis of C. acuminata. One possible explanation for the different patterns of expression is that cis elements required for the appropriate expression of CaTSB1 in roots lay outside the promoter region. We replaced the nopaline synthase terminator in the 893-bp CaTSB1 promoter::GUS construct with a 1-kb DNA fragment from the 3′ end of the untranslated region of the CaTSB1 gene. This substitution had no effect on the spatial
The expression pattern in roots (not shown). The appropriate cis elements may have been lying within the transcribed region, further upstream than –893, or perhaps a root-specific trans-acting factor required for expression in *C. acuminata* is absent in tobacco.

**DISCUSSION**

The predicted protein encoded by *CaTSB1* shared high similarity to the previously reported TSB proteins from both maize and Arabidopsis. The N terminus of the gene had an 18% Ser-plus-Thr content, a feature found in plastid transit peptides. All enzymes of the Trp biosynthetic pathway, including TSB, have been reported to occur in the chloroplasts in Arabidopsis (Zhao and Last, 1995), but tissue prints of *C. acuminata* and expression of promoter::GUS fusions in transgenic tobacco plants showed that most TSB expression was in the vascular tissues of the shoot and subepidermal cortex of the roots, where plastids do not differentiate into chloroplasts. In fact, there appeared to be little expression in tissues where photosynthetically active chloroplasts were present.

We were surprised to find expression of an amino acid biosynthetic enzyme limited to specific tissues. The pea gene *GS3A*, which encodes a cytosolic form of Gln synthetase, was also expressed exclusively in vascular tissue,
but other genes of this family were expressed in photosynthetically active tissues (Edwards et al., 1990). It is possible that TSB, encoded by CaTSB1 or another TSB gene, was expressed at levels sufficient to maintain metabolism within all cells but too low to be detected by tissue printing.

In the shoots of transgenic tobacco plants, the pattern of TSB promoter activity correlated well with the expression patterns seen in C. acuminata. Histochemical GUS staining was seen mainly in the vascular tissues of stems, petioles, and young leaves. Tobacco plants are not known to produce indole alkaloids; therefore, the expression patterns seen here were intrinsic to the promoter and were not due to induction by increased demand for secondary products. Promoter deletion analysis showed that, in comparison to the 896-bp promoter, the truncated promoters drove expression that was quantitatively diminished but spatially similar. No GUS expression was seen in plants containing the two promoters shorter than 110 bp (Fig. 6). It is possible that all of the cis elements for the spatial and developmental expression in the shoot were present within the 110-bp CaTSB1 promoter region, and the sequences further upstream simply affected expression quantitatively.

Expression of the GUS fusions in tobacco roots was variable, a result also seen with fusions of the Arabidopsis TSB promoters in transgenic Arabidopsis (Pruitt and Last, 1993). Our tissue-printing results indicated that in C. acuminata TSB expression was high in the outer cortex of the root and low in the central vascular tissue (Fig. 4). In tobacco, GUS staining was seen in the root apical and lateral meristems and in the regions around the lateral root-branching sites. We did not observe GUS staining in the vascular tissue or the epidermis of tobacco roots. A similar expression pattern in transgenic Arabidopsis roots was reported by Pruitt and Last (1993) with the Arabidopsis TSB1 promoter::GUS fusion. Twelve of their 19 TSB1::GUS transgenic lines showed expression in root apical meristems. An inconsistent and nonuniform GUS stain was observed in root tissue (other than root tips) in 8 of their 19 transgenic lines. Stress-induced expression of the TSB1 promoter expression was eliminated as a possible source of variable expression in Arabidopsis.

Placing the CaTSB1 promoter in a heterologous system may have been the cause for the inconsistent expression in tobacco roots, but this should not have been the reason for variable expression of the Arabidopsis TSB1 promoter expressed in Arabidopsis. It is likely that the cis elements required for correct expression of TSB in roots lay outside the promoter region used in gene fusions from both Arabidopsis and C. acuminata. Sequences within introns of phosphoribosylanthranilate transferase, another Trp pathway gene, are required for high-level expression of the GUS reporter gene, particularly in roots (Rose and Last, 1997). Although additional regulatory regions probably lay outside the sequence included in our promoter fusions, expression of GUS from 893 bp of the promoter region was remarkably similar to the pattern of TSB expression seen in C. acuminata.

Although we isolated only a single TSB gene from C. acuminata, the unexplained XmnI band on the Southern blot (Fig. 2) suggests that there may have been at least one uncloned TSB gene in the genome. If there were other TSB genes, CaTSB1 was by far the most highly expressed gene during early seedling development. Because the pattern of expression of the CaTSB1 promoter in transgenic tobacco plants correlated with total TSB expression in C. acuminata shoots, any other TSB genes must have been regulated similarly or expressed at a very low level. The discrepancy between the expression of CaTSB1 in tobacco roots and total TSB expression in C. acuminata roots could be due to an uncloned, root-specific TSB gene, although neither of the two Arabidopsis TSB genes were reliably expressed in transgenic Arabidopsis roots (Pruitt and Last, 1993).

Expression of TSB throughout C. acuminata plants correlated well with organs that accumulated high concentrations of camptothecin. The shoot apex, young leaves, and bark had high levels of TSB and camptothecin, whereas older leaves, axillary buds, and roots had lower levels of TSB and camptothecin (Fig. 3; López-Meyer et al., 1994). C. acuminata seeds also had high concentrations of camptothecin (López-Meyer et al., 1994). When the seeds were allowed to imbibe, camptothecin levels briefly declined and then transiently increased during seedling growth (López-Meyer and Nessler, 1997). The accumulation of camptothecin in young seedlings may represent a defense mechanism for this vulnerable stage in the plant’s life cycle. TSB was expressed at its highest levels shortly after germination (Fig. 5), before the concentration of camptothecin peaks at d 10 (López-Meyer and Nessler, 1997). If the pattern of TSB expression were unique to indole alkaloid-producing plants, this would suggest that TSB expression was responding to the demand for Trp for alkaloid production. On the other hand, if a similar pattern of expression is found in nonalkaloid-producing plants, this would suggest that indole alkaloid metabolism has developed in locations that provide its precursors. The correlation between sites of TSB expression in C. acuminata and the sites of expression of the CaTSB1 promoter in tobacco favors the latter scenario.

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LITERATURE CITED


