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Tissue-Specific Expression of the β-Subunit of Tryptophan Synthase in *Camptotheca acuminata*, an Indole Alkaloid-Producing Plant[1]

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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 Trp 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.

Trp provides the indole moiety for monoterpene indole alkaloid biosynthesis. Trp is decarboxylated by TDC to produce tryptamine. Tryptamine is then conjugated to the terpenoid secologanin, 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 Trp biosynthesis and the indole alkaloid pathway are coordinately regulated.

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

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 N_2 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 3×10^5 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 *Eco*RI/*Bgl*II 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 5×10^5 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 *Sac*I/*Hin*dIII 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 *Bgl*II/*Hin*dIII 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 *Xba*I. 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 *Hin*dIII 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 *Sac*I/*Hin*dIII fragment from the TSB cDNA was subcloned into the expression vector pET23a(+) (Novagen, Madison, WI). After insertion of the cDNA, the *Nco*I 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

PVDF membrane. The blot was first blocked with 5% dry milk in TTBS buffer (0.05% Tween 20, 20 mM Tris, and 500 mM NaCl, pH 7.5) for 1 h and then incubated with anti-TSB antiserum at 1:3000 dilution at room temperature overnight. TSB was detected by alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody (Bio-Rad) or ^{125}I-protein A. TSB protein was quantified from ^{125}I-treated blots with a Fujix BAS 2000 Bio-Imaging Analyzer (Fuji).

Tissue Printing

C. acuminata tissues were hand-sectioned with double-edged steel blades (Ted Pella, Redding, CA) and printed on a nylon membrane as described by Ye and Varner (1991). The membranes were then treated as protein blots for western blotting. Anti-TSB antiserum (1:3000 dilution) was used as the primary antibody and alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (1:3000 dilution) was used as the secondary antibody. As a negative control, preimmune serum at a 1:3000 dilution was used to replace the anti-TSB antibody in parallel prints. To observe the anatomical structure, tissue sections were stained with toluidine blue.

DNA Construction and Tobacco Transformation

The 893-bp promoter fragment and its deletions were generated by PCR, the fragments of which were confirmed by sequencing. For each promoter, the upstream primer was from the specific deletion region of the TSB promoter. The downstream primer 5′-GTAAACAGCC**ATG**GCTT-GAG-3′ was common for all promoter deletions and mutated at two residues near the ATG start site (indicated by bold type) to create an *Nco*I site. The PCR fragments were ligated into pBluescript SK+ at the *Eco*RV site. Promoter sequences (as *Hin*dIII-*Nco*I fragments) were transferred from the resulting plasmids into the pNco-GUS vector (C.L. Nessler, unpublished construction). These manipulations resulted in the promoters being translationally fused with the GUS reporter gene, followed by a nopaline synthase terminator. The promoter::GUS constructs were then subcloned into the binary vector pBI101 and transferred to the *Agrobacterium tumefaciens* LB4404 strain for leaf disc transformation of tobacco (Horsch et al., 1985).

Quantitative GUS Assay and Histochemistry

Transgenic tobacco tissues were collected and frozen in liquid N_2. Protein was extracted by grinding the tissue in extraction buffer (50 mM NaPO4, pH 7.0, 10 mM EDTA, 0.1% Sarkosyl, 0.1% Triton X-100, and 10 mM β-mercaptoethanol), and protein concentration was determined by using a Bradford assay kit (Bio-Rad). Quantitative GUS assays were performed as described by Jefferson (1987). Fluorescence was measured by a fluorometer (model DyNA Quant200, Hoefer, San Francisco, CA). A standard curve was made from a series of dilutions of 4-methylumbelliferone (Sigma). Histochemical localization of GUS activity was performed by incubating tissues with 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (Sigma) in 100 mM sodium phosphate buffer. After 3 to 16 h of incubation at 37°C, tissues were cleared with 70% ethanol and dissected for photography. All photographs were taken with a stereomicroscope (model SZH10, Olympus) on Ektachrome Tungsten 160 color film (Kodak).

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 fractionations 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 (*Eco*RI/*Bgl*II) 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 6461 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

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1  MA.....A.-R.................---P.......P.P..R.......
2  ..VYTNP.C..TNTSAFPGPYRPYSN....SRFSFNLDKFRP.TSAIKVP
3  ---------.-PGPPP----------...---------.A.EG.RRRGRGR
4  ---------.-----------------...---------------------
5  ..TASTA.TF.PS---SVSASSELTHLRS.SKLPKFT.L.SA.SRSSSSF
6  ..ASGTS.TF.ASVSSAPSSSSQLTHLKS.FKAVKYT.L.SS.SKSSS-F

1  S..CTIA.....--------..G..P...QRPDSFGRFGKFGGKYVPET
2  .IC....REMEKER.......-SERE.DVL.....................
3  NAA---GQAVAAEASPAAVEMGN.AAAPGL....AM....R.........
4  --------------.....-------------------.R.........
5  .VS....KDPAVVMADSEKIKAA.SD.TMW.....................
6  .VS....KDPPVLM.......AA.SD.ALW.....................

1  LMHALTELESAF.ALATDD.FQKELDGILKDYVGRESPLYFAERLTEHYK
2  ..Y.........RS.SG.QV.........................L...
3  ............H......E..............................
4  .........N..H......E..............................
5  .....S...T..YS....ED..R..AE.....................R
6  .....S......Y......D..R..A.......................R

1  R.NGEGPLIYLKREDLNHTGAHKINNAVAQALLAKRLGKKRIIAETGAGQ
2  .P.....E...........................................
3  .AD.T..............................Q..............
4  .AD.T.............R.....................Q..........
5  .E.................................................
6  .E.................................................

1  HGVATATVCARFGLQCIIYMGAQDMERQALNVFRMRLLGAEVRAVHSGTA
2  ................V..................................
3  .........R.........................................
4  ...................................K..............
5  ..........................................G.......
6  ..............E...........................G.......

1  TLKDATSEAIRDWVTNVETTHYILGSVAGPHPYPMMVREPHAVIGKETRK
2  ..................S.................................
3  ..........................................K.......R
4  ..........................................K.......R
5  ....................................D..............
6  ....................................D..............

1  QAMEKWGGKPDVLVACVGGGSNAMGLFHEFV.D.DVRMIGVEAAGFGLDS
2  ..L.............................D.K................
3  ...D............................E.Q...LV.L....H.V.T
4  ...H............................E.Q...L.......H.V.T
5  ................................D.TE................
6  ..L.............................N.TE................

1  GKHAATLTKG.VGVLHGAMSYLLQDDDGQIIEPHSISAGLDYPGVGPEHS
2  ..........E........................................
3  D.........Q......S...........V......................
4  D.........Q......S...........V......................
5  ..........D........................................
6  ..........D........................................

1  FLKDIGRAEY.SVTDEEALEAFKRVSRLEGIIPALETSHALAYLEKLCPT
2  ..........YCC...........L.................F.......
3  ..........D....Q...D...............................
4  ..........D....Q...D...............................
5  ....V.....F................................H.......
6  .F..M.....Y.I......................................

1  LPDG.RVVLNCSGRGDKDVHTA.KYLDV
2  ..N.TK.................I.H.Q.
3  .A..V...V..............S.....
4  ....V..................S.....
5  ....A.....F.........Q..I...E.
6  .S..T.....F.........Q.VA....
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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.

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 *Xmn*I band, were consistent with the restriction pattern of the *CaTSB1.* This *Xmn*I 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.

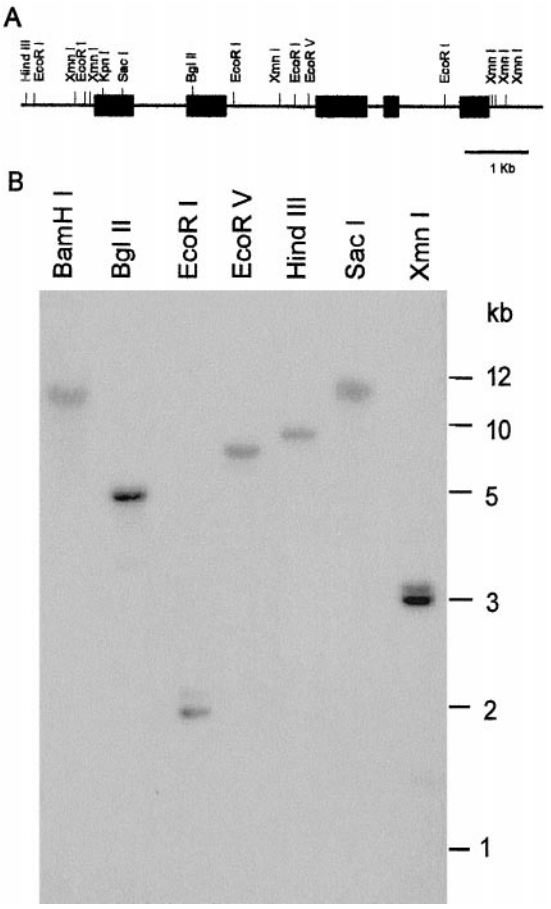

Figure 2. Genomic Southern analysis of TSB sequences. A, Restriction map of *CaTSB1*. B, Autoradiograph of a genomic Southern blot probed with a 933-bp *Sac*I-*Hin*dIII fragment from the center of the coding region of the TSB cDNA. This fragment contains part of exon 1 and all of exons 2 and 3. The *Hin*dIII 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-^{32}P]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 *Xmn*I 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 (but probably not the tracheary elements), and primary 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*.

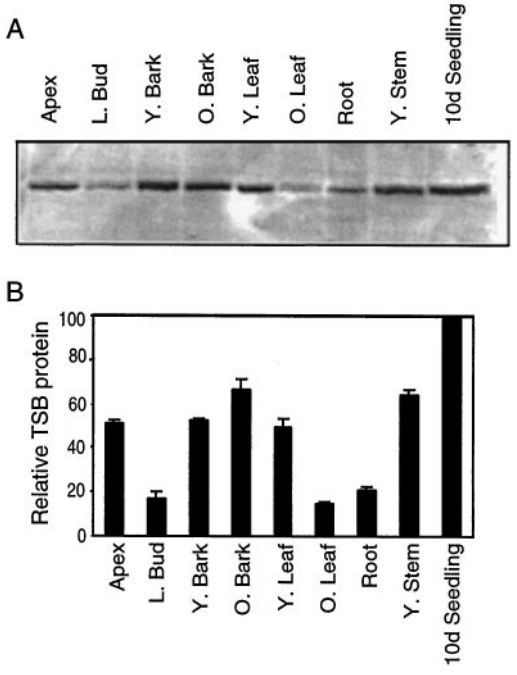

Figure 3. Expression of TSB protein in *C. acuminata* tissues. A, Immunoblot with antibody to TSB. Each lane contained 20 μg of total protein from apex (Apex), lateral bud (L. Bud), young bark (Y. Bark), old bark (O. Bark), young leaf (Y. Leaf), old leaf (O. Leaf), root (Root), young stem (Y. Stem), or 10-d-old seedlings (10d Seedling). The samples were separated on a 10% SDS-polyacrylamide gel, blotted onto a PVDF membrane, and probed with anti-TSB antibody and ^{125}I-labeled Protein A. B, Quantitative analysis of TSB protein. Relative amounts of protein detected on the blot were quantified with a Fujix BAS 2000 Bio-Imaging Analyzer.

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 *CaTSB1* 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 *CaTSB1* mRNA peaked earlier than *TDC1* and camptothecin accumulation (d 10 and 12 postimbibition, respectively). The accumulation of *CaTSB1* 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 *CaTSB1*::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

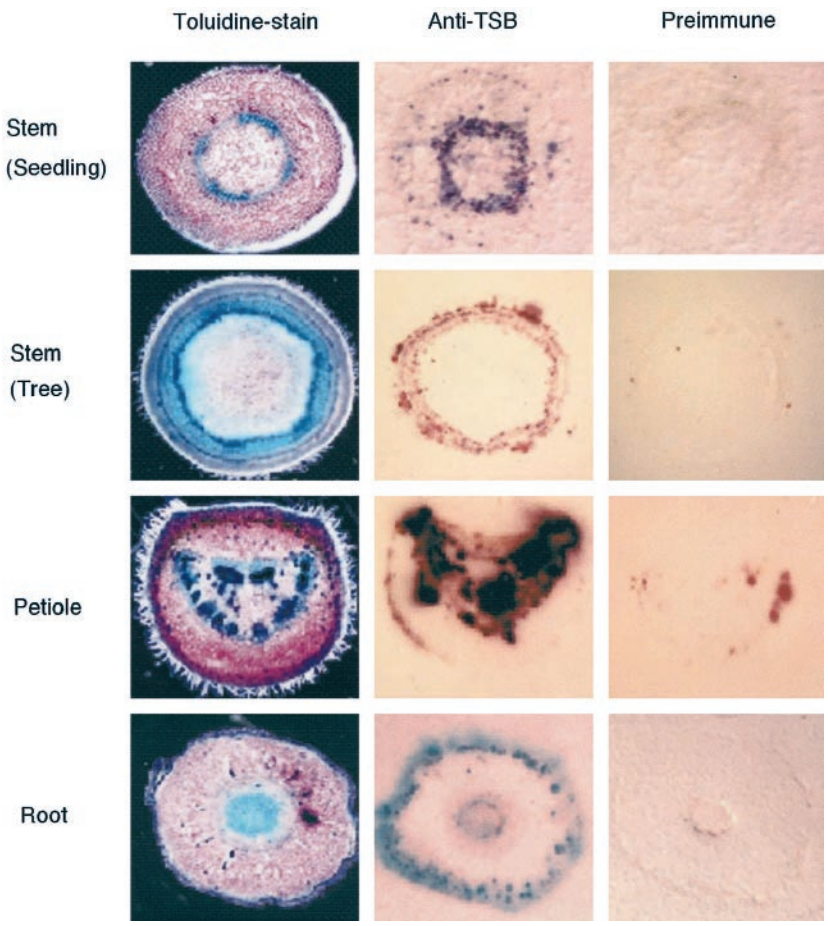

Figure 4. Localization of TSB protein in *C. acuminata* tissues by tissue printing. Cross-sections of *C. acuminata* were stained with 0.025% toluidine blue to show the anatomical structure in the left panel. The corresponding tissue prints were probed with anti-TSB antiserum (center) and preimmune serum, followed by alkaline phosphatase-conjugated goat anti-rabbit IgG (right).

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

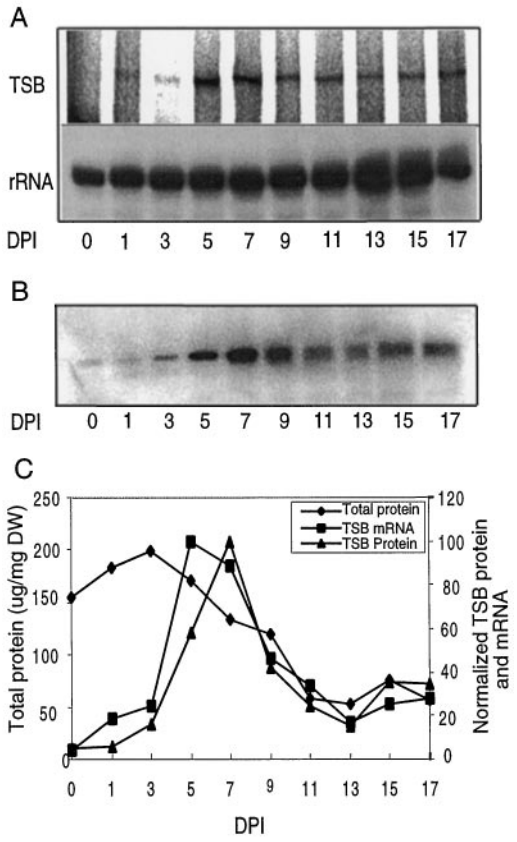

Figure 5. Expression of TSB during *C. acuminata* seedling development. A, Expression of *CaTSB1* mRNA. Total RNA was extracted from d-0 (dried seeds) to d-17 seedlings. *CaTSB1* mRNA was detected by RNase protection assays. B, Expression of TSB protein. Total protein (20 μg protein/lane) from d-0 to d-17 seedlings was separated on a 7.5% SDS-PAGE gel, blotted onto a PVDF membrane, and probed with anti-TSB antisera and ^{125}I-labeled Protein A. C, Total protein change and quantitative analysis of TSB expression during seedling development. Relative amounts of mRNA and protein from the blots in A and B were quantified with a Fujix BAS 2000 Bio-Imaging Analyzer and expressed as a percentage of the level from the highest expressing sample. DPI, Days postimbibition; DW, dry weight.

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

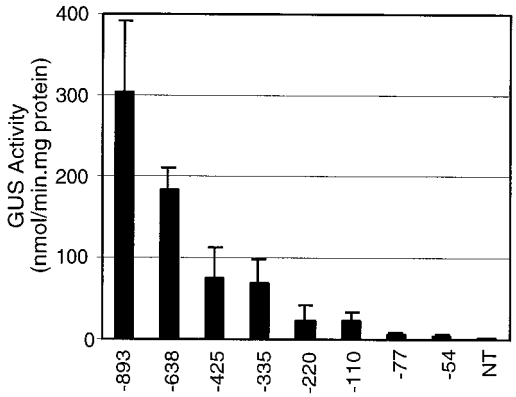

Figure 6. Expression of *CaTSB1* promoter::GUS fusions in transgenic tobacco. The numbers on the *x* axis represent the length of the promoter sequences driving GUS expression. All promoters were translationally fused to the GUS start codon. GUS activity from 6-d-old transgenic tobacco seedlings was measured with the fluorogenic substrate 4-methylumbelliferone glucuronide and expressed as nanomoles of 4-methylumbelliferone produced per minute per milligram of protein. For each deletion, three plants from each of five independently transformed lines were assayed. Error bars represent the SD. NT, Nontransformed control.

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,

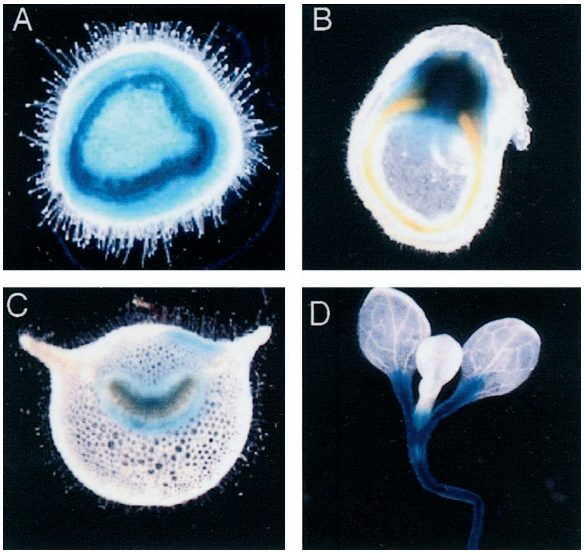

Figure 7. Histochemical analysis of GUS activity in transgenic tobacco plants harboring the 893-bp *CaTSB1* promoter::GUS construct. A, Cross-section of young stem with staining in vascular cylinder. B, Cross-section of older stem through a node, with staining only in the vascular tissue associated with new growth in the lateral bud. C, Cross-section of petiole with very light staining in the vascular region. D, Six-day-old tobacco seedling with staining in the hypocotyl and at the base of cotyledons.

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 *Xmn*I 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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