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Expression of enzymatically active and correctly targeted strictosidine synthase in transgenic tobacco plants

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Abstract. Strictosidine, a precursor to over 1000 indole alkaloids including the anti-tumor drugs vinblastine, vincristine, and camptothecin, is produced by the condensation of tryptamine and secologanin. Strictosidine synthase, the enzyme responsible for this condensation, is the first committed step in the indole-alkaloid pathway. We have introduced a modified cDNA encoding strictosidine synthase from *Catharanthus roseus* (L.) Don. (McKnight et al. 1990, Nucl. Acids Res. **18,** 4939) driven by the CaMV 35S promoter into tobacco (*Nicotiana tabacum* L.). Transgenic tobacco plants expressing this construct had from 3 to 22 times greater strictosidine-synthase activity than *C. roseus* plants. Ultrastructural immunolocalization demonstrated that strictosidine synthase is a vacuolar protein in *C. roseus* and is correctly targeted to the vacuole in transgenic tobacco. Immunoblot analysis of strictosidine synthase showed that two distinct forms of the enzyme were produced in transgenic tobacco plants but that only a single form was made in *C. roseus*. This observation indicates that the second form of the protein is not simply a result of overexpression in tobacco, but may reflect differences in protein processing between tobacco and *C. roseus*.

Key words: Camptothecin – *Catharanthus* – Indole alkaloids – *Nicotiana* (transgenic) – Protein processing and targeting – Strictosidine synthase – Vacuole (strictosidine synthase)

Introduction

Over 1000 indole alkaloids, including quinine, strychnine, and the anti-cancer compounds vinblastine, vincristine, and camptothecin are derived from strictosidine (reviewed by Cordell 1974). Stöckigt and Zenk (1977) demonstrated that crude extracts from *Catharanthus roseus* suspension cells could couple tryptamine and secologanin to form strictosidine, as shown in Fig. 1. Subsequent work lead to the isolation of cDNAs encoding strictosidine synthase from *Rauvolfia serpentina* (Kutchan et al. 1988) and from *C. roseus* (McKnight et al. 1990).

Vinblastine and vincristine are produced from strictosidine by a complex pathway (reviewed by Cordell 1974), and camptothecin is produced from strictosidine by a separate and much shorter pathway of four or five reactions (Carte et al. 1990). Because complex biochemical pathways are often regulated at early stages, overexpression of strictosidine synthase, the first enzyme committed to indole-alkaloid biosynthesis, may allow manipulation of the alkaloid pathway to understand its control and thereby alter the abundance and spectrum of alkaloids produced in transgenic plants.

Because transformation of apocynaceous plants is not yet routine, we have expressed the *C. roseus* strictosidine-

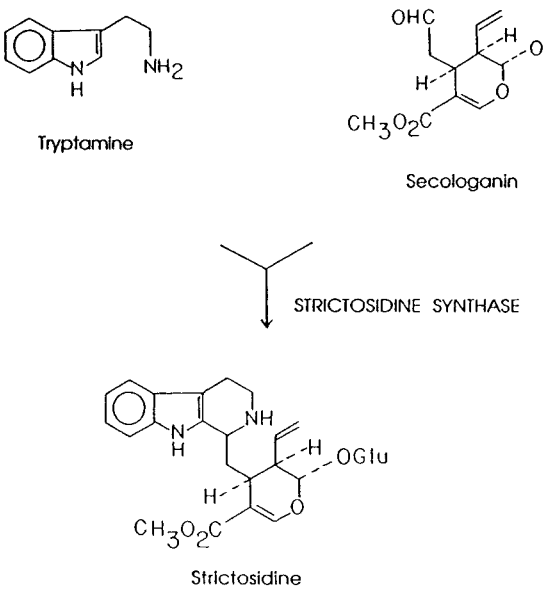

Fig. 1. Biosynthesis and structure of strictosidine

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Abbreviations: cDNA = complementary DNA; TLC = thin-layer chromatography

synthase cDNA sequence from the CaMV 35S promoter in transgenic tobacco to determine whether functional enzyme could be produced at high levels. In this report, we show that strictosidine synthase encoded by a modified cDNA sequence is expressed in an enzymatically active form in transgenic tobacco plants, and that this protein is targeted to the vacuole in tobacco, just as it is in *C. roseus*. Transgenic tobacco plants, however, also produce a second form of strictosidine synthase. The novel form appears to have a lower molecular weight, and it does not appear to be the result of overexpression in transgenic plants. The production of two forms of strictosidine synthase indicates differences in protein processing between *C. roseus* and tobacco.

Materials and methods

Strictosidine synthase constructions. The isolation and sequence of a cDNA clone encoding strictosidine synthase from *C. roseus* has been described (McKnight et al. 1990). The coding region was isolated from the bacteriophage λ clone by polymerase chain reactions with primers designed to be complementary to the open reading-frame boundaries and carrying restriction-enzyme cleavage sites (BamHI in the 5′ primer and SacI in the 3′ primer). The resulting DNA fragment was subcloned in pBI121 (Jefferson et al. 1987) in place of the deleted β-glucuronidase gene. This subcloning resulted in placement of the strictosidine-synthase coding-region between the CaMV 35S promoter and the nopaline synthase poly-A addition site. This construction is referred to as SS2. An additional construction, SS3 (Fig. 2), was made in a similar fashion, except that DNA encoding an additional nine amino acids from the signal peptide of *Rauvolfia serpentina* strictosidine synthase (Kutchan et al. 1988) was added to the 5′ end of the *C. roseus* cDNA. Restriction enzymes were purchased from Boehringer Mannheim (Indianapolis, Ind., USA) and Taq polymerase used in the PCR reactions was from Promega (Madison, Wis., USA). Oligonucleotides were synthesized by the Advanced DNA Technologies Laboratory of Texas A&M University.

Transformation procedures. Plasmids were mobilized into the disarmed *Agrobacterium tumefaciens* strain LBA4404 by triparental mating (Matzke and Matzke 1986). Tobacco (*Nicotiana tabacum* L. cv. Xanthi) leaves were transformed by the leaf-disk procedure of Horsch et al. (1985), except that nurse cultures were not used. Transformed shoots were rooted in 0.5 MS salts (Murashige and Skoog 1962) containing 100 μg kanamycin/ml and 100 μg cefotaxime/ml. Transgenic tobacco plants were maintained in a greenhouse.

Antibody production. An *Eschericia coli* strain containing a plasmid with the mature strictosidine-synthase coding region fused to the *tac* promoter was created and supplied by Dr. C.A. Roessner (Department of Chemistry, Texas A&M University). The cells were grown at 37° C in LB medium (Maniatis et al. 1982) to 0.5 OD_{600} and induced with 2 mM *iso*propylthio-β-D-galactoside (Sigma Chemical Co., St. Louis, Mo. USA) for 2 h. The cells were harvested and stored at −20° C. Strictosidine synthase aggregated into insoluble granules in the induced cells. These granules were purified from lysed cells by repeated washes in buffer containing increasing amounts of urea (Schoner et al. 1985). Strictosidine synthase was injected into rabbits to produce antibody.

Protein gel blot analysis and immunolocalization. Proteins were extracted from young tobacco leaves by grinding tissue in an equal volume of β-glucuronidase extraction buffer (Jefferson et al. 1987). The proteins then were resolved on 10% polyacrylamide-sodium dodecyl sulfate (SDS) gels and electroblotted onto polyvinylidene-difluoridide membranes (Matsudaira 1987). Strictosidine synthase was detected by incubating the blot in anti-strictosidine synthase antiserum followed by goat anti-rabbit immunoglobulin G antibody conjugated to alkaline phosphatase and reacted with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium as described by the supplier (BioRad, Richmond, Cal., USA).

The on-grid immunolocalization procedures of VandenBosch (1986) were used to localize strictosidine synthase in tobacco and *C. roseus* root cells.

Enzyme assays. Strictosidine-synthase assays were performed as described by Eilert et al. (1987). Briefly, plant extracts were in-

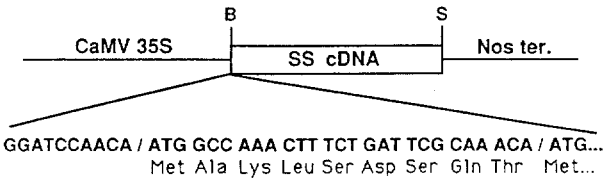

Fig. 2. Construction of SS3 gene. The *C. roseus* strictosidine synthase cDNA (McKnight et al. 1990) was extended by the polymerase chain reaction to include a BamHI restriction site at the 5′ end of the coding region and an additional nine codons based upon the *R. serpentina* strictosidine-synthase cDNA (Kutchan et al. 1988). The resulting nucleotide sequence of the 5′ region of SS3 is indicated. This modified cDNA was then inserted between the BamHI and SacI sites (indicated by *B* and *S*, respectively) of pBI121 (Jefferson et al. 1987). This subcloning placed the strictosidine-synthase coding region between the CaMV 35S promoter and the nopaline synthase poly-A addition site *(nos-ter)* as indicated in the figure. The SS2 gene was constructed in a similar manner except that the *C. roseus* cDNA was not extended, and the coding region begins with the last methionine codon shown

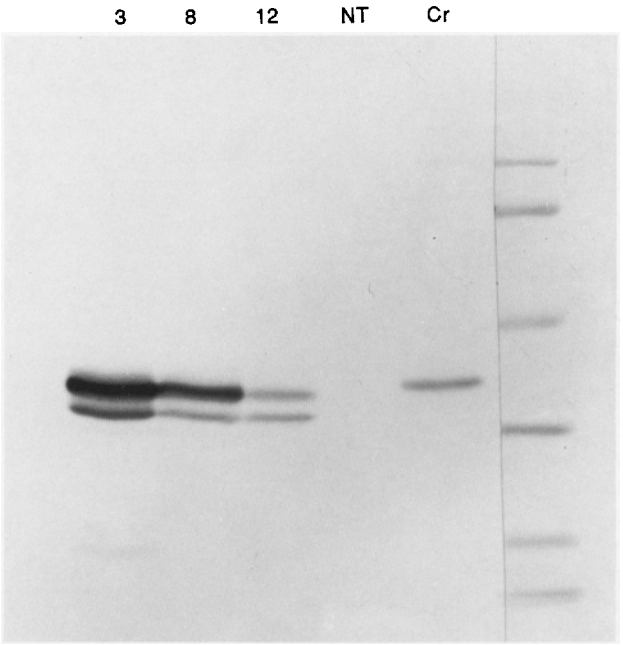

Fig. 3. Immunodetection of strictosidine synthase in *C. roseus* and transgenic tobacco. Fifty micrograms of protein from each plant was loaded per lane. From the left the lanes contain protein from transformants 3, 8, and 12, non-transformed tobacco, and *C. roseus*. The far-right lane contains molecular-weight standards of 97.4, 66.2, 45.0, 31.0, 21.5 and 14.4 kDa

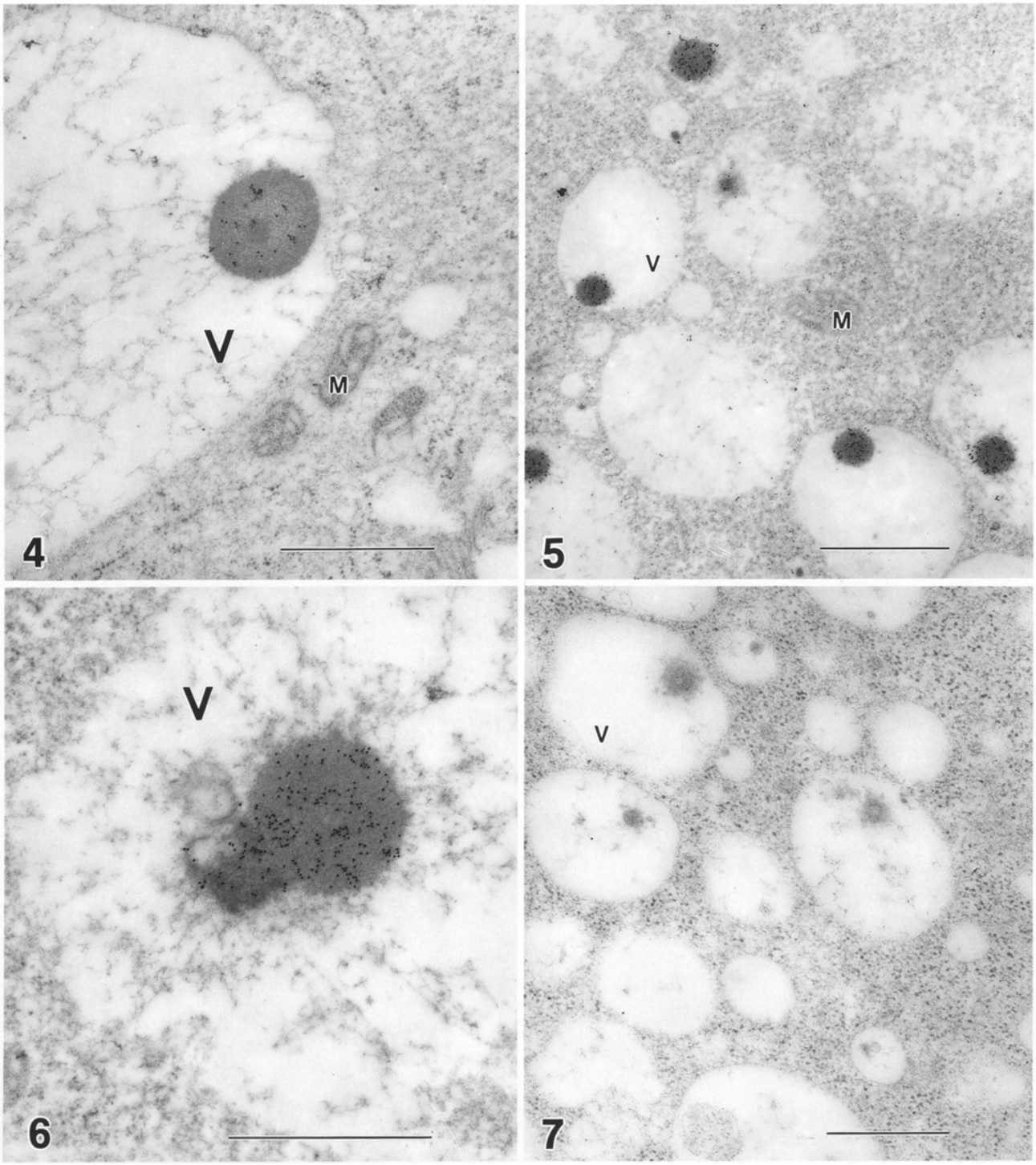

Fig. 4–7. Immunolocalization of strictosidine synthase in root cells of *C. roseus* and transgenic tobacco. V = vacuole, M = mitochondria. Bars = 0.5 μm. **Fig. 4** *C. roseus*; **Figs. 5, 6** transgenic tobacco plant No. 5, showing most of the gold within relatively electron-dense inclusion bodies; **Fig. 7** untransformed tobacco control

cubated with secologanin (Aldrich, Milwaukee, Wis., USA) and C^{14}-labeled tryptamine (New England Nuclear, Boston, Mass. USA), and the alkaloids were extracted from the reaction by ethyl acetate and separated on Silica gel 60 thin-layer chromatography (TLC) plates (E. Merck, Darmstadt, FRG). Enzyme activity was quantified by illuminating the TLC plates with 360-nm ultraviolet light, scraping the strictosidine spot into scintillation fluid, and determining the amount of radioactivity.

Results

We could not detect expression of strictosidine synthase, by immunoblot analysis nor by enzyme assay, in any of 17 plants transformed with the SS2 construction. This result may be because the N-terminal region of the signal peptide of the *C. roseus* cDNA lacks a positively charged

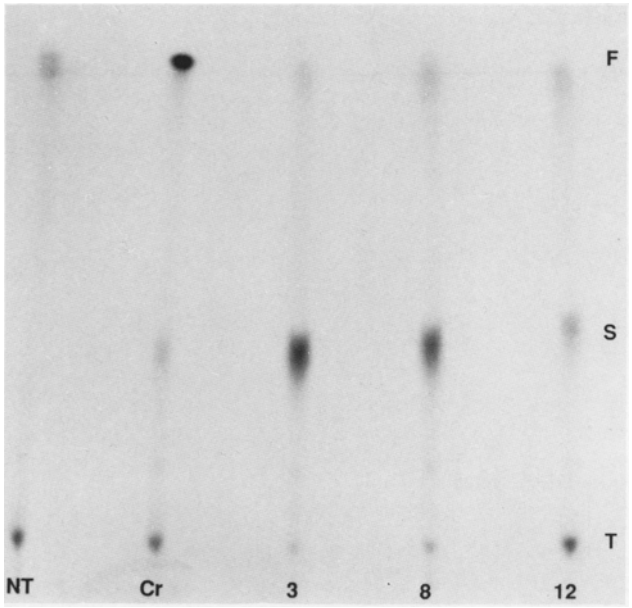

Fig. 8. Autoradiogram of TLC assay for strictosidine synthase activity. Secologanin and ^{14}C-labeled tryptamine were incubated with protein extracts. Alkaloid products were extracted, fractionated by TLC, and the TLC plate was exposed to X-ray film for 3 d. From the left, the reactions contained extracts from non-transformed tobacco, *C. roseus*, and transgenic plants No. 3, 8 and 12. *T*, *S*, and *F* indicate, in this sequence, the positions of tryptamine, strictosidine, and the solvent front

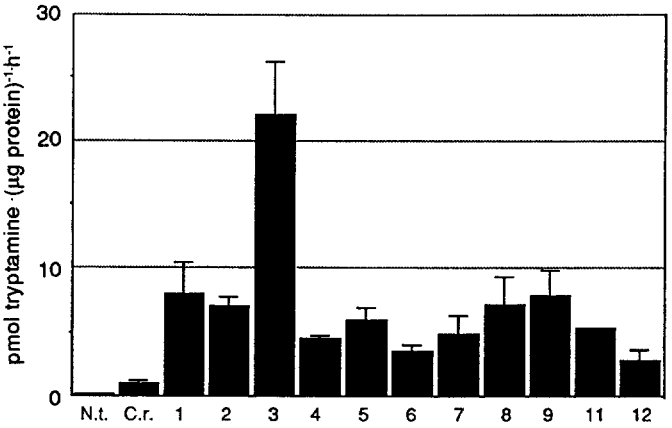

Fig. 9. Strictosidine-synthase activity in transgenic tobacco plants. Bars indicate strictosidine-synthase activity levels (tryptamine incorporated) in non-transformed *Nicotiana tabacum (N.t.)*, *C. roseus (C.r.)*, and transgenic SS3 tobacco plants *(1–12)*. Means ± SD

amino acid which may be required for efficient secretion across membranes (reviewed by Boyd and Beckwith 1990). We have not determined whether this deficiency is a consequence of recovering a truncated cDNA, or is actually a feature of the *C. roseus* strictosidine signal peptide.

The SS3 construction was created to overcome this apparent deficiency by the addition of the first nine amino acids of the signal peptide from *Rauvolfia serpentina* strictosidine synthase, including the positively charged lysine residue at amino-acid position 3 (see Fig. 2). Immunoblot analysis showed that strictosidine synthase was produced at high levels in plants containing the SS3 construction (Fig. 3). As expected, the level of expression in transgenic tobacco was higher than that found in *C. roseus* (Fig. 3; also see Figs. 4–9). One difference in expression of strictosidine synthase between tobacco and *C. roseus* is the presence of a lower-molecular-weight form in tobacco (Fig. 3). This new form may be processed differently from the *C. roseus* form, or it may be a degradation product.

Immunogold labeling was used to localize strictosidine synthase in transgenic tobacco plants. In both *C. roseus* and transgenic tobacco plants, the enzyme was localized almost exclusively to the vacuole (Figs. 4–7). Further localization within the vacuole was also evident, since most of the gold label was associated with relatively electron-dense inclusion bodies. We have not yet determined whether these electron-dense regions correspond to an actual feature of the vacuole, or whether they are artifacts created during fixation, but they seem to be less dense and less common in vacuoles of untransformed plants.

In addition to being targeted to the correct subcellular compartment in transgenic tobacco, the protein was also enzymatically active. Protein extracts from *C. roseus* plants, untransformed tobacco plants, and transgenic SS3 plants were incubated with secologanin and C^{14}-labeled tryptamine, and the reaction products were separated by TLC. Autoradiography of the TLC plates showed the production of a radioactive alkaloid that migrated with the correct R_f for strictosidine by extracts from *C. roseus* and transgenic SS3 plants, but not from untransformed tobacco (Fig. 8). The radioactivity migrating with the solvent front in the *C. roseus* lane of Fig. 8 did not appear in most assays, and there was no correlation between the appearance of this product(s) and the amount of strictosidine produced.

The identity of strictosidine produced in the assay was confirmed by converting it to strictosidine lactam with 10 N NaOH. Following TLC, the plates were stained with ceric ammonium sulfate, a reagent which stains strictosidine lactam yellow (Farnsworth et al. 1964). Extracts from all eleven transgenic tobacco plants and *C. roseus* produced an alkaloid that co-migrated with authentic strictosidine lactam standard (provided by Dr. J. Balsevich, Plant Biotechnology Institute, Saskatoon, Sask., Canada) and stained yellow when sprayed with ceric ammonium sulfate (data not shown).

Quantitation of tryptamine incorporated into strictosidine showed that the SS3 plants expressed 3–22 times as much enzymatic activity as *C. roseus* plants (Fig. 9). The levels of activity correlate well with the level of strictosidine synthase detected on Western blots (Fig. 3).

Discussion

Manipulation of secondary-product synthesis in transgenic plants may be useful for augmenting naturally occurring plant defense compounds, increasing the production of plant-derived pharmaceuticals, and decreasing toxic metabolites in food crops.

In this report we have demonstrated that the first enzyme committed to indole-alkaloid production, strictosidine synthase, can be expressed in an active and correctly targeted form in transgenic tobacco plants. The specific activities of strictosidine synthase in transgenic tobacco were 3–22 times greater than in *C. roseus* plants.

Strictosidine synthase has been produced in *E. coli* from the *Rauvolfia serpentina* cDNA, but most of the protein was found in insoluble inclusion bodies (Kutchan 1989). In addition to this result, there are other reasons to think that strictosidine synthase produced in bacteria differs substantially from that produced in plants. Post-translational modifications that could differ between strictosidine synthase in plants and in bacteria include correct cleavage of the signal peptide and any potential propeptide, the formation and arrangement of disulfide bonds, N-linked glycosylation, and the overall conformation of the protein.

Although transgenic tobacco plants produce high levels of enzymatically active and correctly targeted strictosidine synthase, they differ from *C. roseus* plants in that the protein is found in two forms. One form is electrophoretically similar to that found in *C. roseus*, and the other migrates somewhat faster. The faster-migrating form could represent a novel glycoform, an alternatively processed polypeptide, or a degradation product of the *C. roseus*-like form. Whatever its origin, this novel form probably indicates differences in protein processing between *C. roseus* and tobacco since it is proportionally less in transgenic plants expressing higher amounts of strictosidine synthase (see Fig. 3) and is therefore unlikely to be an artifact of overexpression.

Both substrates for strictosidine synthase apparently are absent from tobacco plants. In their study of transgenic tobacco plants expressing tryptophan decarboxylase, Songstad et al. (1990) detected little, if any, tryptamine in untransformed tobacco plants. We found that extracts from transgenic tobacco plants expressing strictosidine synthase do not incorporate radioactive tryptamine into strictosidine nor any other detecable compound (data not shown), indicating that if secologanin is found in tobacco at all, it must be rare. Although neither substrate for strictosidine synthase is present in untransformed tobacco, transgenic tobacco plants that express tryptophan decarboxylase accumulate high levels of tryptamine (Songstad et al. 1990). Thus, when the limiting enzymes for secologanin biosynthesis are cloned it may be possible to reconstitute the pathway for monoterpenoid indole alkaloids up to strictosidine in a completely heterologous species.

In spite of recent advances in plant biotechnology, it still remains prohibitively expensive to produce most natural products in cultured plant cells. Overexpression of key enzymes in transgenic cultures may allow economically feasible production of secondary products under controlled, in-vitro conditions. If the high levels of expression of strictosidine synthase and tryptophan decarboxylase achieved in tobacco can be duplicated in indole-alkaloid-producing plants, the production of the antitumor alkaloids vinblastine, vincristine, and camptothecin may be increased.

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