Plant Physiol. (1990) 94, 1410–1413
0032-0889/90/94/1410/04/$01.00/0

Received for publication April 30, 1990
Accepted July 20, 1990

High Levels of Tryptamine Accumulation in Transgenic Tobacco Expressing Tryptophan Decarboxylase[1]

David D. Songstad[2], Vincenzo De Luca[3], Normand Brisson[4], Wolfgang G. W. Kurz, and Craig L. Nessler[5]*

Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, Saskatchewan S7N 0W9, Canada

ABSTRACT

A full-length complementary DNA clone encoding tryptophan decarboxylase (TDC; EC 4.1.1.28) from *Catharanthus roseus* (De Luca V, Marineau C, Brisson N [1989] Proc Natl Acad Sci USA 86: 2582–2586) driven by the CaMV 35S promoter was introduced into tobacco (*Nicotiana tabacum*) to direct the synthesis of the protoalkaloid tryptamine from endogenous tryptophan. Young, fully expanded leaves of CaMV 35S-TDC transformed plants had from four to 45 times greater TDC activity than did controls. Tryptamine accumulated in transgenic plants to levels that were directly proportional to their TDC specific activity. Despite their increased tryptamine content, the growth and development of the CaMV 35S-TDC plants appeared normal with no significant differences in indole-3-acetic acid levels between high tryptamine and control plants. Plants with the highest TDC activity contained more than 1 milligram of tryptamine per gram fresh weight, a 260-fold increase over controls.

The enzymology associated with biosynthesis of alkaloids is the subject of much study. It is generally accepted that protoalkaloid production is the first committed step in most alkaloid pathways. In the production of indole and isoquinoline alkaloids, the protoalkaloids tryptamine and tyramine are important precursors. Both are created through the enzymatic decarboxylation of amino acids: tryptophan to form tryptamine and tyrosine to form tyramine (12).

Tryptamine has also been suggested as a possible precursor for IAA[6] biosynthesis (1). This has not been firmly established, however, because of the small tryptamine pool size found in plants such as *Zea mays* (6). Nevertheless, it must be recognized that any attempt to elevate tryptamine levels for increased alkaloid synthesis might also result in additional auxin production affecting normal patterns of growth and development.

De Luca *et al.* (5) reported the isolation of a full-length cDNA coding for TDC (EC 4.1.1.28) from *Catharanthus roseus*. Because this clone was found to function in *Escherichia coli* (5), we assumed that it would also function in heterospecific transgenic plants when placed under appropriate transcriptional control. In this report we describe the expression of a CaMV 35S promoter-TDC cDNA chimeric gene in transgenic tobacco and the accumulation of high amounts of tryptamine in these plants. Auxin levels in a high tryptamine and a control plant are also compared to determine if the increased tryptamine pool results in elevated levels of IAA.

MATERIALS AND METHODS

Plasmid Construction and Plant Transformations

The full-length TDC cDNA insert (5) was subcloned 3′ to the CaMV 35S promoter of pBI121 (8) in place of the deleted β-glucuronidase gene. The plasmid, pBI121, a derivative of the binary vector Bin19 (2), contains a selectable marker gene that confers kanamycin resistance upon transformed plant cells. The resulting plasmid, CaMV 35S-TDC, was mobilized into the disarmed *Agrobacterium tumefaciens* strain LBA4404 by triparental mating (14).

Tobacco (*Nicotiana tabacum* L. cv Xanthi) leaves were transformed by the leaf disc method (7) with either CaMV 35S-TDC or pBI121 as a control. Transformed shoots were rooted in 1:2 Murashige-Skoog salts (15) containing 100 μg/mL kanamycin.

Analysis of TDC Activity, Tryptamine, and Auxin

Crude protein extracts were prepared by homogenizing 0.5 g of leaf tissue in 1.25 mL of extraction buffer (0.1 M Hepes, pH 7.5; 1 mM dithiothreitol) followed by desalting using a Sephadex G-25 column. TDC activity was measured by the conversion of L-[methylene-^{14}C]tryptophan to [^{14}C]tryptamine according to the method of De Luca *et al.* (4). Protein was quantitated by the dye-binding method of Bradford (3).

For identification and quantification of primary amines, tobacco leaves (1 g/plant) were extracted with absolute methanol containing 0.5% concentrated HCl. Extracts were diluted by the addition of 10% (v/v) H_2O and passed through a Sep-

[1] National Research Council of Canada No. 31492.

[2] Present address: Monsanto Company, 700 Chesterfield Village Parkway, Chesterfield, MO 63198.

[3] Permanent address: Jardin Botanique de Montreal, 4101 rue Sherbrooke Est, Montreal, PQ H1X 2B2, Canada.

[4] Permanent address: Departement de Biochimie, Université de Montreal, Montreal PQ H3C 3J7, Canada.

[5] Permanent address: Department of Biology, Texas A&M University, College Station, TX 77843-3258.

[6] Abbreviations: IAA, indole-3-acetic acid; CaMV 35S, cauliflower mosaic virus 35S promoter; CTAB, hexadecyltrimethyl ammonium bromide; TDC, tryptophan decarboxylase; SSC, standard saline citrate.

Pak C_{18} cartridge (Millipore Corp., Milford, MA) to remove pigments.

Twenty-μL aliquots of each extract were examined by liquid chromatography on a reverse phase C-8 column at 35°C using a discontinuous water-methanol gradient. Each solvent included 0.1 M ammonium acetate. The gradient was run at a flow rate of 2 mL/m as follows (H_2O:MeOH):95:5 from 0 to 2 m, 20:80 from 2 to 14 m, 95:5 from 14 to 14.5 m, and 95:5 from 14.5 to 18 m. Compounds were identified by comparing retention times and absorption spectra as determined by a diode array, with those of authentic standards.

Indole-3-acetic acid was analyzed using an Phytodetek IAA monoclonal antibody kit (Idetek Inc., San Bruno, CA). Approximately 1 g of leaf material was collected from control plants transformed with pBI121 and CaMV 35S-TDC transgenic plants. The extraction, preparative, and quantitative methods recommended by the kit manufacturer were followed and IAA samples were assayed in triplicate over a 1000-fold dilution series. According to data supplied by the manufacturer, major cross-reactants for the IAA antibodies include: indole-3-acetylglycine (57.9%), indole-3-acrylic acid (5.5%), and indole-3-acetone (5.2%). Other conjugates tested showed ≥1.5% cross-reactivity.

Nucleic Acid Purification and Analysis

DNA and RNA were extracted from tobacco leaf tissue by the CTAB procedure and separated on a cesium chloride step gradient (16). For slot blots, total RNAs (20 μg) were denatured in 50% formamide, 7% formaldehyde, and 1 × SSC (150 mM NaCl, 15 mM sodium citrate) at 68°C for 15 min, mixed with 2 volumes of 20 × SSC, and applied to nitrocellulose (13). Genomic DNA (10 μg) was digested with *Eco*R I and subjected to electrophoresis through a nondenaturing agarose gel. The gel was alkali-treated and neutralized, and the DNA was blotted to MagnaGraph nylon membrane (Micron Separations Inc., Westborough, MA) according to the manufacturer's instructions. Hybridizations of the RNA slot blot and DNA gel blot were performed with random-primed ^{32}P-labeled probe in 50% formamide and 5 × SSC at 42°C. The stringency of the final washes was 1 × SSC at 65°C.

RESULTS

Approximately 100 shoots were regenerated from inoculated tobacco leaf segments and subsequently rooted in the presence of 100 μg/mL kanamycin. Fifty CaMV 35S-TDC R_0 plants were transferred to soil and grown in a greenhouse. Leaf samples were collected from each plant and assayed for TDC activity. Preliminary screening of these plants showed elevated TDC activity in approximately 80% of the putative TDC transformants, with the levels ranging from eight- to 85-fold greater than controls (data not shown).

Although the CaMV 35S promoter is customarily regarded as constitutive, it is known to be more active in younger, more vigorously growing regions of transgenic tobacco plants (18). We therefore examined the effect of age on CaMV 35S-driven TDC expression in the plant that had shown the highest TDC activity in our initial screening.

Table I. *Differential TDC Activities in Leaf and Bud Tissues of CaMV 35S-TDC and Control Transgenic Tobacco Plants*

Tissue[a]	Plant	TDC Activity[b]
Flower bud	Control	2.3 ± 0.23
	T-201-1	108.7 ± 29.74
Leaf 1	Control	1.8 ± 0.18
	T-201-1	70.4 ± 4.79
Leaf 2	Control	1.0 ± 0.03
	T-201-1	58.0 ± 1.06
Leaf 3	Control	0.8 ± 0.23
	T-201-1	39.1 ± 6.58
Leaf 4	Control	1.5 ± 0.20
	T-201-1	28.3 ± 5.10
Leaf 5	Control	1.3 ± 0.03
	T-201-1	24.5 ± 5.85

[a] About 0.4 g of flower bud or leaf tissue was collected for each TDC assay. Leaf 1 (youngest) through leaf 5 (oldest) denote nodes from the floral axis. [b] pmol tryptophan converted to tryptamine μg^{-1} protein h^{-1}.

This plant, T-201-1, displayed a gradient of TDC activity along the axis ranging from over 100 pmol of tryptophan converted to tryptamine per μg protein per h in the flower bud to less than 25 pmol converted in older, nonsenescent leaf tissue (Table I). In view of these data, TDC activities in each plant were reanalyzed using the youngest fully expanded leaf (Table II). A 10-fold variation in TDC activity was again observed between the highest and lowest TDC expressers, though the absolute increase in TDC activity was reduced from eight- to 85-fold to from four- to 45-fold greater than controls.

Fourteen plants (13 CaMV 35S-TDC plus one pBI121 control) were selected for additional analyses from among those showing low, intermediate, and high levels of TDC activity. Tryptamine was extracted from the youngest fully expanded leaf of each plant, quantitated by liquid chromatography and compared with previously measured TDC activities (Table III). Regression analysis of these data describes a linear relationship between TDC activity and tryptamine content with an r^2 value of 0.77 (Fig. 1).

TDC copy number was estimated in each of the 14 transformants in a reconstruction blot using a 1.6 kilobase *Eco*R I fragment from the TDC cDNA as a reference. Every TDC transformant, except one, appeared to have incorporated only one copy of the CaMV 35S-TDC insert (data not shown). The multicopy plant, T-183-1, contained at least five copies of the TDC construct, yet it was among the plants with the lowest TDC activity and tryptamine (Table 3).

The relative abundance of steady-state TDC mRNA in each transformant was compared by applying equal loads of total RNAs (20 μg/plant) extracted from young, fully expanded leaves to nitrocellulose and probing the slot blot with ^{32}P-labeled TDC insert. An autoradiogram of this blot shows that transformants with higher TDC activity generally have more TDC mRNA than plants with lower TDC activity (Fig. 2).

To investigate possible effects of elevated tryptamine on auxin synthesis, IAA was measured in plant T-201-1, the transformant with the highest tryptamine content as well as

Table II. *TDC Activity in the Youngest, Fully Expanded Leaves of Control and CaMV 35S-TDC Transgenic Tobacco Plants*

Plant	TDC Activity[a]
Control	1.3
Control	1.5
T-150-3	5.8
T-196-1	6.1
T-181-1	6.6
T-157-1	13.1
T-97-1	13.4
T-183-1	13.6
T-162-1	15.1
T-105-1	15.1
T-145-2	15.6
T-178-1	16.1
T-186-1	17.2
T-177-2	18.4
T-180-1	19.2
T-36-1	19.4
T-210-1	21.4
T-159-4	23.7
T-128-1	25.2
T-194-1	29.5
T-177-1	29.5
T-118-1	29.8
T-156-1	30.5
T-117-2	32.0
T-157-2	32.3
T-193-1	33.3
T-152-1	35.3
T-142-2	36.1
T-164-4	36.1
T-213-1	37.1
T-153-1	39.4
T-148-1	40.9
T-162-3	41.6
T-37-1	42.6
T-122-2	42.9
T-200-1	44.9
T-121-2	51.2
T-28-1	51.7
T-153-2	59.0
T-147-3	59.8
T-201-1	64.3

[a] pmol tryptophan converted to tryptamine μg^{-1} protein h^{-1}.

Table III. *Comparison of TDC Activity and Tryptamine Content in Young Leaves of Control and CaMV 35S-TDC Transgenic Tobacco Plants*

Plant	TDC Activity[a]	Tryptamine (μg/g fresh wt)	Blot Coordinates[b]
Control	1.3	4	A1
T-150-3	5.8	247	A2
T-97-1	13.4	344	A3
T-183-1	13.6	357	A4
T-105-1	15.1	497	A5
T-186-1	17.2	480	A6
T-180-2	19.2	310	B1
T-210-1	21.4	667	B2
T-128-1	25.2	345	B3
T-118-1	29.8	637	B4
T-157-2	32.3	401	B5
T-148-1	40.9	1077	B6
T-162-3	41.6	906	C1
T-201-1	64.3	1085	C2

[a] pmol tryptophan converted to tryptamine μg^{-1} protein h^{-1}. [b] See Figure 2.

DISCUSSION

Data obtained in this investigation demonstrate that transgenic plants can be directed to synthesize and accumulate high levels of secondary metabolites without apparent adverse effects. Insertion of the TDC cDNA into tobacco under control of the CaMV 35S promoter resulted in an up to 45-fold increase in TDC activity and a 260-fold increase in tryptamine accumulation. The 10-fold variation in expression observed among individual TDC transformants is similar to that reported for other genes driven by the CaMV 35S promoter (11) and can be attributed primarily to position effects (17) rather than differences in gene copy number.

Despite the large amounts of tryptamine accumulated in some individuals, all CaMV 35S-TDC plants were fertile and appeared morphologically normal throughout their development. The similar IAA levels in control and high tryptamine plants suggest that, at least in tobacco, tryptamine pool size may not have a significant influence on IAA synthesis. It is the greatest TDC activity. In a control tobacco plant carrying the CaMV 35S-β-glucuronidase construct there was approximately 4.2 μg tryptamine/g fresh weight and 291 ± 39 pmol IAA/g fresh weight in young, fully expanded leaves. This compares with 1085 μg tryptamine/g fresh weight and 305 ± 24 pmol IAA/g fresh weight in the leaf of T-201-1. Thus, although the tryptamine pool in the TDC plant was over 260 times larger than the control, their IAA contents were comparable, with less than 4% difference between them. This result is also consistent with the normal appearance and lack of any morphological abnormalities observed among the high tryptamine plants.

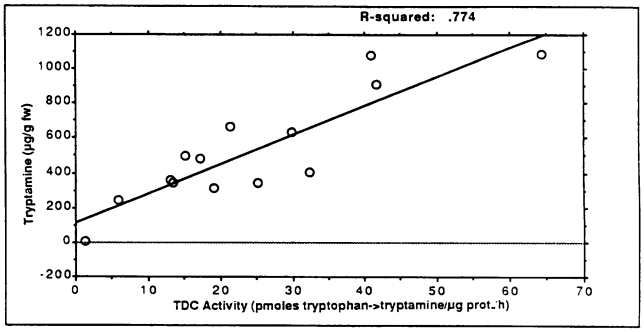

Figure 1. Regression analysis showing a linear relationship between TDC activity and tryptamine content in transgenic tobacco. Data points plotted from columns 1 and 2 of Table III.

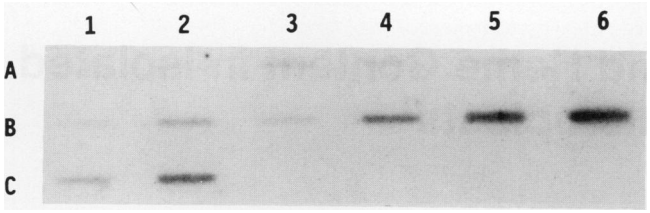

Figure 2. Comparison of relative TDC mRNA levels in control and CaMV 35S-TDC transformants. Autoradiogram of an RNA slot blot probed with a ^{32}P-labeled, 1.6 kilobase *Eco*R I fragment from the TDC cDNA. Twenty μg of total RNAs from young, fully expanded leaves from each transformant were applied per slot. Samples were applied in order of increasing TDC activity as listed in Table III.

possible, however, that the tryptamine synthesized in these plants is quickly sequestered (*i.e.* in the vacuole) and therefore not available for auxin synthesis. Subcellular localization of tryptamine in these transgenic plants should help resolve this question.

The ability of tobacco to grow and develop normally in the presence of high levels of tryptamine suggests that the CaMV 35S-TDC construction might be also useful in the genetic engineering of other species, including *Catharanthus roseus*. Overexpression of TDC in this species may increase metabolite flow toward the synthesis of the valuable antitumor alkaloids vinblastine and vincristine. As genes for other enzymes in the indole alkaloid pathway become available, such as the cDNA for strictosidine synthase recently cloned from *Rauvolfia serpentina* (9, 10), unique opportunities will arise for directing secondary metabolism through gene transfer technology.

ACKNOWLEDGMENTS

The authors gratefully recognize the skilled technical assistance of John Magus and Brock Chatson in performing the TDC enzyme assays and tryptamine quantification.

LITERATURE CITED

1. **Bandurski RS, Nonhebel HM** (1984) Auxins. *In* MB Wilkins, ed, Advanced Plant Physiology, Pitman Publishing Inc., London, pp 1–20
2. **Bevan M** (1984) Binary *Agrobacterium* vectors for plant cell transformation. Nucleic Acids Res **12:** 8711–8721
3. **Bradford MM** (1976) A rapid and sensitive method for quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal Biochem **72:** 248–254
4. **De Luca V, Alvarez-Fernandez F, Campbell D, Kurz WGW** (1988) Developmental regulation of enzymes of indole alkaloid biosynthesis in *Catharanthus roseus*. Plant Physiol **86:** 447–450
5. **De Luca V, Marineau C, Brisson N** (1989) Molecular cloning and analysis of cDNA encoding a plant tryptophan decarboxylase. Comparison with animal dopa decarboxylases. Proc Natl Acad Sci USA **86:** 2582–2586
6. **Epstein E, Cohn JD, Bandurski RS** (1980) Concentration and metabolic turnover of indoles in germinating kernels of *Zea mays* L. Plant Physiol **65:** 415–421
7. **Horsch RB, Fry JE, Hoffmann NL, Eichholtz D, Rogers SG, Fraley RT** (1985) A simple and general method for transferring genes into plants. Science (Washington DC) **227:** 1229–1231
8. **Jefferson RA, Kavanagh TA, Bevan MW** (1987) GUS fusions. β-Glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J **6:** 3901–3907
9. **Kutchan TM, Hampp N, Lottspeich F, Beyreuther K, Zenk MH** (1988) The cDNA clone for strictosidine synthase from *Rauvolfia serpentina*. DNA sequence determination and expression in *Escherichia coli*. Fed Eur Biochem Soc **237:** 40–44
10. **Kutchan TM** (1989) Expression of enzymatically active cloned strictosidine synthase from the higher plant *Rauvolfia serpentina* in *Escherichia coli*. FEBS Lett **257:** 127–130
11. **Lagrimini LM, Bradford S, Rothstein S** (1990) Peroxidase-induced wilting in transgenic tobacco plants. The Plant Cell **2:** 7–18
12. **Luckner M** (1984) Secondary Metabolism in Microorganisms, Plants and Animals, Ed 2. Springer-Verlag, New York
13. **Maniatis T, Fritsch EF, Sambrook J** (1982). Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
14. **Matzke AJM, Matzke MA** (1986) A set of novel Ti plasmid derived vectors for the production of transgenic plants. Plant Mol Biol **7:** 357–365
15. **Murashige T, Skoog F** (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant **15:** 473–497
16. **Taylor B, Powell A** (1982) Isolation of plant DNA and RNA. Focus **4:** 4–6
17. **Weising K, Schell J, Kahl G** (1988) Foreign genes in plants. Transfer, structure, expression and applications. Annu Rev Genet **22:** 421–478
18. **Williamson JD, Hirsch-Wyncott ME, Larkins BA, Gelvin SB** (1989) Differential accumulation of a transcript driven by the CaMV 35S promoter in transgenic tobacco. Plant Physiol **90:** 1570–1576