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Overexpression of a tryptophan decarboxylase cDNA in *Catharanthus roseus* crown gall calluses results in increased tryptamine levels but not in increased terpenoid indole alkaloid production

OSCAR J.M. GODDIJN[1‡], ED J.M. PENNINGS[2], PETER van der HELM[1], ROB A. SCHILPEROORT[1], ROB VERPOORTE[2] and J. HARRY C. HOGE[1*]

[1]*Institute of Molecular Plant Sciences, Leiden University, Clusius Laboratory, PO Box 9505, 2300 RA Leiden, Netherlands*
[2]*Center for Bio-Pharmaceutical Sciences, Leiden University, Division of Pharmacognosy, Gorlaeus Laboratory, PO Box 9502, 2300 RA Leiden, Netherlands*

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The enzyme tryptophan decarboxylase (TDC) (EC 4.1.1.28) catalyses a key step in the biosynthesis of terpenoid indole alkaloids in *C. roseus* by converting tryptophan into tryptamine. Hardly any *tdc* mRNA could be detected in hormone-independent callus and cell suspension cultures transformed by the oncogenic T-DNA of *Agrobacterium tumefaciens*. Supply of tryptamine may therefore represent a limiting factor in the biosynthesis of alkaloids by such cultures. To investigate this possibility, chimaeric gene constructs, in which a *tdc* cDNA is linked in the sense or antisense orientation to the cauliflower mosaic virus 35S promoter and terminator, were introduced in *C. roseus* cells by infecting seedlings with an oncogenic *A. tumefaciens* strain. In the resulting crown gall tumour calluses harbouring the *tdc* sense construct, an increased TDC protein level, TDC activity and tryptamine content but no significant increase in terpenoid indole alkaloid production were observed compared to empty-vector-transformed tumour calluses. In tumour calluses containing the *tdc* antisense construct, decreased levels of TDC activity were measured. Factors which might be responsible for the lack in increased terpenoid indole alkaloid production in the *tdc* cDNA overexpressing crown gall calluses are discussed.

Keywords: *Catharanthus roseus*; tryptophan decarboxylase; terpenoid idole alkaloids; *tdc* cDNA overexpression; crown gall calluses

Introduction

A characteristic feature of plants is their capacity to produce an impressive variety of secondary metabolites, which have no recognized role in plant cell growth and differentiation but serve, among other functions, in plant defence against pathogenic and herbivorous organisms. Many of these compounds are also of pharmacological and industrial interest (Phillipson, 1990). The production of valuable secondary metabolites using plant cell cultures has been the aim of many studies in the past decade (Hay *et al.*, 1988). Despite much effort, cell cultures have proved to be a poor system for the biosynthesis of economically relevant amounts of these compounds. It is becoming increasingly clear that this, at least in part, is due to the strict regulatory control(s) imposed on the activity of secondary metabolism in plant cells.

The regulation of terpenoid indole alkaloid (TIA) biosynthesis in *C. roseus*, a member of the Apocynaceae family, is of current interest. *C. roseus* has the capacity to form, among other alkaloids, the monomeric TIAs ajmalicine and serpentine, which are used in the treatment of cardiac and circulatory diseases, as well as the dimeric TIAs vincristine and vinblastine, which both have potent anticancer properties. A key enzyme in the

‡Present address: Mogen International NV, Einsteinweg 97, 2333 CB Leiden, Netherlands.
*To whom correspondence should be addressed.

biochemical pathway which leads to their synthesis is tryptophan decarboxylase (TDC) (EC 4.1.1.28), as it links primary metabolism to TIA production by converting tryptophan into tryptamine. Tryptamine is subsequently condensed with the iridoid secologanin by the enzyme strictosidine synthase (SSS) (EC 4.3.3.2) to form the glucoalkaloid strictosidine, the universal precursor for all known TIAs found in plants.

Considerable progress has been made in the isolation of genes encoding key-enzymes involved in TIA biosynthesis and their regulation. A cDNA clone encoding TDC was isolated from *C. roseus* (DeLuca *et al.*, 1989) and subsequently its corresponding gene (Goddijn *et al.*, 1994). The regulation of *tdc* expression was studied in *C. roseus* (Goddijn *et al.*, 1992; Pasquali *et al.*, 1992) and *Nicotiana tabacum* (Goddijn *et al.*, 1994) and its overexpression in *N. tabacum* (Songstad *et al.*, 1990; Goddijn *et al.*, 1993; Poulsen *et al.*, 1994) and *Peganum harmala* (Berlin *et al.*, 1993). A number of cDNA clones encoding SSS were isolated from *Rauvolfia serpentina* (Kutchan *et al.*, 1988) and *C. roseus* (McKnight *et al.*, 1990; Pasquali *et al.*, 1992) and expression studies were carried out in *C. roseus* plants and cell suspensions (Pasquali *et al.*, 1992; Roewer *et al.*, 1992). The availability of genes encoding key enzymes in TIA biosynthesis makes it feasible to perform experiments designed to enhance this biosynthetic pathway.

In cell cultures of *C. roseus*, the formation of TIAs seems to be incompatible with active cell proliferation (Knobloch and Berlin, 1980). This observation and similar observations in cell cultures of other species have led to the use of separate media for cell growth or the production of secondary metabolites (Berlin *et al.*, 1983). Production media are characterized by high levels of sucrose, the absence of auxin and cytokinin and low levels of nitrogen and phosphate to reduce cell proliferation. In *C. roseus* suspension cultures, the levels of the TIAs ajmalicine and serpentine are enhanced by transferring cells to production medium (Knobloch *et al.*, 1981). This treatment also induces the translatable *tdc* mRNA level (Noé and Berlin, 1985). Conversely the addition of auxin to hormone-starved cells has been shown to repress the accumulation of *tdc* mRNA at the transcriptional level (Goddijn *et al.*, 1992). In phytohormone-independent cultures of *C. roseus*, established by transformation with *Agrobacterium tumefaciens*, hardly any *tdc* mRNA was detectable and the production of terpenoid indole alkaloids was also very low. These data suggest that the strict regulation of *tdc* expression at least in part accounts for the low level of TIA biosynthesis in *C. roseus*. Other reports suggest that the secologanin pathway has an important regulatory role in TIA biosynthesis. Feeding of secologanin to *C. roseus* suspension cultures results in enhanced alkaloid formation in contrast to the feeding of tryptophan (Merillon *et al.*, 1986, 1989).

We have analysed here whether lack of TDC activity could be a major factor in the limitation of TIA formation in *C. roseus* crown gall calluses. The *tdc* coding region was coupled in both sense and antisense orientation to the 35S CaMV promoter and terminator and introduced in *C. roseus* cells using an oncogenic *A. tumefaciens* strain. Effects on TDC protein levels, TDC activity, tryptamine content and TIA production in the resulting crown gall tumour calluses were examined.

Materials and methods

Plant materials

Cell suspension cultures of *C. roseus* L. (G. Don) were grown in LS medium (Linsmaier and Skoog, 1965), containing 1-naphthaleneacetic acid (NAA) (2 mgl^{-1}), kinetin (0.2 mgl^{-1}) and 3% w/v sucrose, at 27 °C on a Kühner Lab-shaker (orbit of 5 cm) at 95 rpm with a photoperiod of 12 h. Subculturing was performed every 10–12 days by 10-fold dilution of the cells in fresh medium. Induction of TDC activity was achieved by transferring 10-day-old cells to induction medium (IM) (Berlin *et al.*, 1983).

Transformed root cultures of *C. roseus* obtained by infecting aseptic leaves with *A. rhizogenes* strain LBA9402 (S. Hoekstra, unpublished results) were subcultured each week in modified Gamborg B5 medium (Gamborg *et al.*, 1968). The concentration of macronutrients and the $CaCl_2$ concentration were lowered respectively four and two times.

Catharanthus seeds (sold as *Vinca rosea*, variety Morning mist) were obtained from Kieft (Blokker, Netherlands), and plants were grown in a greenhouse at 23 °C.

Isolation of TDC from C. roseus *cell suspension cultures and preparation of antiserum*

TDC was isolated from induced *C. roseus* cell cultures as described previously (Pennings *et al.*, 1989). After size exclusion chromatography (SEC), fractions containing TDC activity were pooled and subjected to preparative SDS-PAGE. After staining, the 47 kDa TDC monomer band was sliced out of the gel and soaked in H_2O. Electroelution of the TDC protein from the gel fragments was performed using ISCO sample concentrators (Hunkapillar *et al.*, 1983). Recovery of the TDC protein after electrophoresis and electroelution was estimated to be 90% via analytical SDS-PAGE.

Eluate samples containing 150 µg of denatured TDC were emulsified with 600 µl of Freund's complete adjuvant (Gibco) and injected subcutaneously in two New Zealand white rabbits. Booster injections, eluate samples containing 75 µg of denatured TDC emulsified with 600 µl of Freund's incomplete adjuvant, were administered three times at two-week intervals. The rabbits were bled two weeks after the last injection. Blood

samples were left at room temperature for several hours and centrifuged at 2000 rpm for 30 min. The clear supernatant was taken and stored at −20 °C. Antibody titres of the two sera were 5×10^{-4} as determined by ELISA.

Construction and screening of a λgt11 expression cDNA library

Total RNA was isolated from a suspension culture of *C. roseus* cultured on induction medium for 24 h. Poly(A)$^+$ RNA was isolated as described (van Slogteren *et al.*, 1983) with a minor modification; binding buffer was used to wash loaded oligo (dT)-cellulose columns. Synthesis of cDNA was essentially as described by Sambrook *et al.* (1989). Phosphorylated *Eco* RI linkers (Pharmacia) were ligated to double-stranded (ds) cDNA, size-selected by chromatography on Sepharose CL-4B and cloned in the *Eco* RI site of λgt11 (Promega). After packaging (Promega packaging mix) and infection of the *E. coli* host strain Y1090 (Promega, $OD_{550} \approx 0.6$, grown in LB containing 0.2% maltose and 10 mM $MgSO_4$) 2.32×10^5 pfu μg^{-1} λgt11 were obtained, of which 82% were recombinant. The cDNA library was amplified according to Huyng *et al.* (1985).

Screening of the library with antiserum raised against denatured TDC was performed according to Promega (Protoblot Immunoscreening System, technical manual). Positive lambda clones were purified and their cDNA inserts (*Eco* RI fragment) were subcloned in pBluescript vectors (Stratagene).

DNA, RNA and protein sequence determination

The cDNA inserts of the pBluescript vectors were sequenced by generating deletions of the *Eco* RI inserts from both 5′ and 3′ ends using the *Exo* III/mung-bean system (Promega). The resulting constucts were sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977). Both strands were sequenced over their entire length. RNA sequencing was performed according to Geliebter (1987). Sequence data were analysed using the University of Wisconsin Genetics Computing Group programs (Devereux *et al.*, 1984). Protein sequencing was performed by Dr D. Choli at the Max Planck Institut für Molekulare Genetik (Berlin, Germany).

Construction of pBDH5, pTDCs and pTDCa

The binary plant expression vector pBDH5 was constructed by deleting the *Sal* I restriction site from a Bin19 binary vector (Bevan, 1984) and inserting a CaMV 35S expression cassette from pDH51 (Pietrzak *et al.*, 1986) as an *Eco* RI fragment (Fig. 3). This construct (pBDH5) in which the orientation of the CaMV 35S promoter and the nopaline synthase (Nos) promoter of the NPTII gene are the same was used in further cloning procedures. An isolated *tdc* cDNA clone was completed by a synthetic *Sal* I-*Eco* RI fragment (see results) which was subcloned in pIC20H (Marsch *et al.*, 1984). The *tdc* cDNA insert was cloned behind this fragment leading to pIST6. The completed *tdc* cDNA was excised as *Sal* I-*Xhol* fragment and cloned in both orientations in the *Sal* I site of the pBDH5 plant expression vector resulting in pTDCs (sense construct) and pTDCa (antisense construct) (Fig. 3). All DNA manipulations were carried out according to Sambrook *et al.* (1989). The constructs were electroporated into *A. tumefaciens* strain LBA1010 (pTiB6 plasmid in a C58-C9 background, (Koekman *et al.*, 1979) as described by Mattonovich *et al.* (1989).

Oncogenic transformation of Catharanthus roseus

Transgenic *C. roseus* calluses were obtained by infecting hypocotyls of three-week-old seedlings with the oncogenic *A. tumefaciens* strain LBA1010 containing the *tdc* constructs. After 3–4 weeks, crown gall tumours were excised and cultured in the absence of phytohormones on LS medium containing kanamycin (100 mg l^{-1}).

RNA isolation and northern blot analysis

Isolation of RNA from plant tissues was performed according to van Slogteren *et al.* (1983). Blotting and hybridization were performed as described previously (Goddijn *et al.*, 1992). Usually, 5–20 μg of total RNA was applied per lane.

Western blot analysis

Proteins were separated by SDS-PAGE using the discontinuous Laemmli system (Laemmli, 1970) and transferred to immobilon PVDF (Millipore) membranes using the Multiphor II Novablot (LKB) electroblotting apparatus. The blot buffer consisted of 50 mM Tris-HCl, 40 mM glycine, 0.04% w/v SDS pH = 8.3 and 20% v/v methanol. Electrophoretic transfer was performed for 2 h at 0.8 mA cm^{-2}. Membranes were rinsed in TBST (1 × TBST = 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) and blocked for 30 min in TBST supplemented with 1% gelatin. Further steps with first and second antibody treatment, washing steps and staining reactions were performed as in the screening procedure used for the λgt11 cDNA library.

Determination of tryptophan decarboxylase activity

TDC activity was determined according to Pennings *et al.* (1987). Protein determinations were performed according to Bradford (1976).

Alkaloid and tryptamine measurements

Alkaloid and tryptamine contents were determined in transgenic *C. roseus* crown gall calluses using a two step extraction method (Schripsema *et al.*, 1992). Freeze-dried cell material (50 mg) was humidified with 0.5 ml

H_2O and extracted with 5 ml dichloromethane. After centrifugation, the dichloromethane fraction was evaporated and the residue was dissolved in 0.5 ml eluent (40 mM $NaH_2PO_4 \cdot H_2O$ pH 3.9, 15% acetonitrile, 0.5% methoxyethanol) and used in HPLC analysis to determine the alkaloid content. Remaining cell material was reextracted after addition of 0.5 ml NaOH with 5 ml dichloromethane to determine the tryptamine content. After centrifugation, the water phase was discarded and the dichloromethane phase was treated as described above. Separation and detection of alkaloids and tryptamine was performed as described by van der Heijden *et al.* (1987).

Results

Transcript levels of tryptophan decarboxylase in C. roseus

Screening of a λgt11 cDNA expression library with antiserum raised against purified, denatured TDC resulted in the isolation of a *tdc* cDNA clone. Subcloning in a Bluescript SK vector yielded plasmid pCCR2. Steady state *tdc* mRNA levels were monitored in several *C. roseus* tissues using the pCCR2 insert as radiolabelled probe (Fig. 1). High *tdc* mRNA levels were detected in the roots of flowering plants grown in a greenhouse.

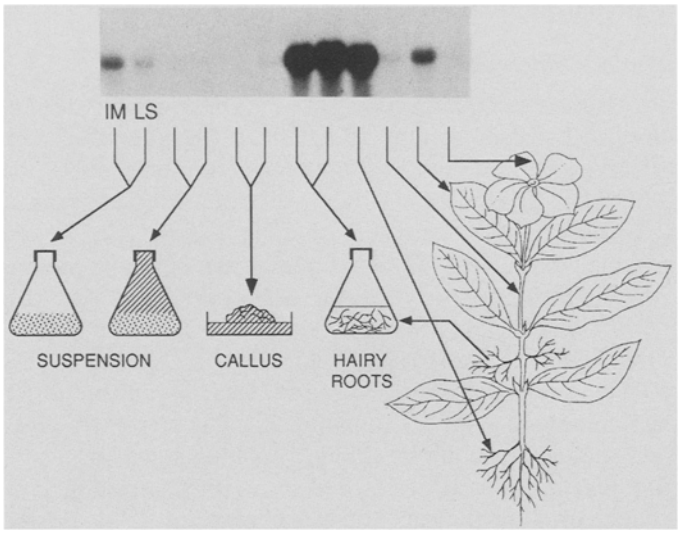

Fig. 1. Steady state *tdc* mRNA levels in cell suspensions, calluses, hairy roots, roots, stems, leaves and flowers of *C. roseus*. Untransformed cell suspension cultures were grown for 7 days on LS or IM medium. Crown gall cell suspension and callus cultures (shaded) transformed by the oncogenic *A. tumefaciens* strain LBA1010 were grown in hormone-free LS medium for 7 days and 4 weeks, respectively. The hairy root cultures, transformed by the *A. rhizogenes* strain LBA 9402, were grown for 4 weeks on modified B5 medium without hormones. Roots, stems, leaves and flowers were taken from plants grown for 3 months in a greenhouse. Equal amounts of total RNA were loaded in each lane.

Similarly high levels were observed in *A. rhizogenes* transformed hairy roots. In leaves, about 5–10-fold lower levels of *tdc* mRNA were found. Very low levels of mRNA were detected in stems and flowers. High, much lower and very low *tdc* mRNA levels were also observed in respectively the roots, leaves and stems of non-flowering plants grown *in vitro* (not shown).

In comparison to root tissue, *tdc* mRNA levels turned out to be at least 10 times lower in untransformed suspension cultures of *C. roseus*. Transfer of these cultures from growth medium (LS) to induction medium (IM), gave rise to about a 5-fold higher steady state level of *tdc* mRNA. This induction is in accordance with the known *de novo* synthesis of tryptophan decarboxylase upon transfer of cell suspensions of *C. roseus* from LS to IM (Noé *et al.*, 1985). Almost no *tdc* mRNA was detected in *A. tumefaciens* transformed crown gall cell suspensions and calluses. Despite the absence of phytohormones in the culture medium, these lines were not able to produce high levels of terpenoid indole alkaloids. This lack of TIA formation could be caused by the low *tdc* transcript levels resulting in low TDC activities and thereby a limiting supply of tryptamine. One option to bypass this limitation is to generate *C. roseus* crown gall calluses harbouring an artificial *tdc* gene consisting of a strong constitutive plant promoter, full-length *tdc* cDNA and a functional plant terminator. By doing so, it is perhaps possible to stimulate the formation of TIAs in crown gall cultures.

Construction of a binary plant expression vector harbouring tdc

Sequence analysis showed that the pCCR2 insert encompasses an open reading frame followed by a stop codon and a poly(A) tail at the 3′ end, but lacked a start codon at the 5′ end. As the insert of pCCR2 represented a partial *tdc* cDNA clone, it was necessary to complete its coding region prior to overexpression in *C. roseus*. Primer extension sequencing of poly(A) RNA revealed the sequence of the missing 5′ part of the open reading frame and two putative translation starts (Fig. 2A).

Determination of the N-terminal amino acid sequence of the purified 47 kDa TDC monomer showed that this protein starts with the amino acid sequence: SPVGEFKPL. This sequence is identical to the amino acid sequence specified by nucleotides 40–67 of the mRNA hybridizing to the pCCR2 insert. The N-terminal amino acid sequence of TDC suggested a processing of the protein coded for by *tdc* mRNA, resulting in deletion of the first 13 amino acids. Based on these data, a 55 bp oligonucleotide flanked by a *Sal* I and a *Eco* RI restriction site, in which the ATG on position +31 serves as translation start, was synthesized to complete the coding region of the pCCR2 insert (Fig. 2B).

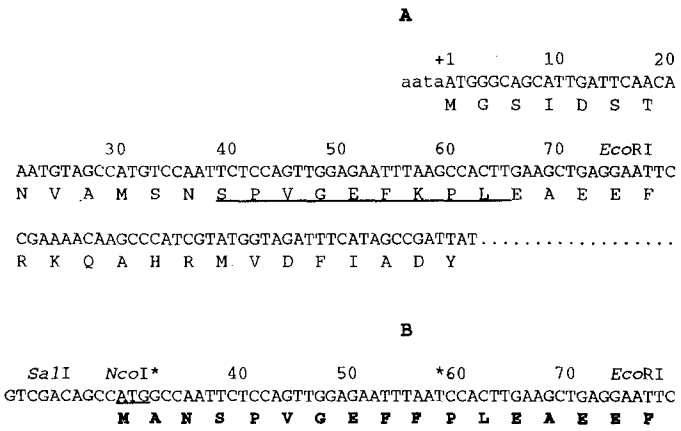

Fig. 2. Nucleotide sequence of the 5′ part of the coding region of full-length *C. roseus tdc* cDNA (Panel A). The predicted amino acid sequence is shown in standard single letter code. The N-terminal amino acids of the purified TDC monomer determined via protein sequencing are underlined. Panel B depicts the synthetic oligonucleotide used to complete our *tdc* cDNA clone. The corresponding amino acid sequence is displayed in bold characters. The ATG which is assumed to be used as translation start is underlined. Two mutations in the sequence resulting from the introduction of a *Nco* I site and a DNA synthesis error are indicated by an asterisk.

Owing to an error in DNA synthesis and the introduction of a *Nco* I site at position +30, expression of the coding region completed by the 55 bp oligoncleotide will result in a TDC monomer starting with the amino acid sequence MANSPVGEFFPL. The synthetically completed *tdc* cDNA sequence was cloned in the sense and antisense orientation between the CaMV 35S promoter and terminator in the binary plant expression vector pBDH5 (Fig. 3) resulting in the plasmids pTDCs (sense) and pTDCa (antisense). The oncogenic *A. tumefaciens* strain LBA1010 was subsequently used to generate kanamycin-resistant and hormone-independent crown gall tumour calluses of *C. roseus* that are transformed with the T-DNAs of pTDCs, pTDCa and pBDH5, respectively.

Analysis of tdc *transgenic* C. roseus *crown gall calluses*

Western blot analysis revealed much higher TDC monomer levels in the pTDCs transformed calluses than in the pBDH5 transformed control calluses (Fig. 4). In pTDCa transformed calluses equal or lower levels of the TDC monomer were detected compared to the control calluses. Relative to the control calluses, substantially increased TDC enzyme activities were detected in all pTDCs transformed calluses, showing that overexpression of our slightly modified *tdc* cDNA coding region results in the synthesis of an active enzyme, which is able to convert tryptophan into tryptamine (Table 1). Only very low or no TDC activity was detected in

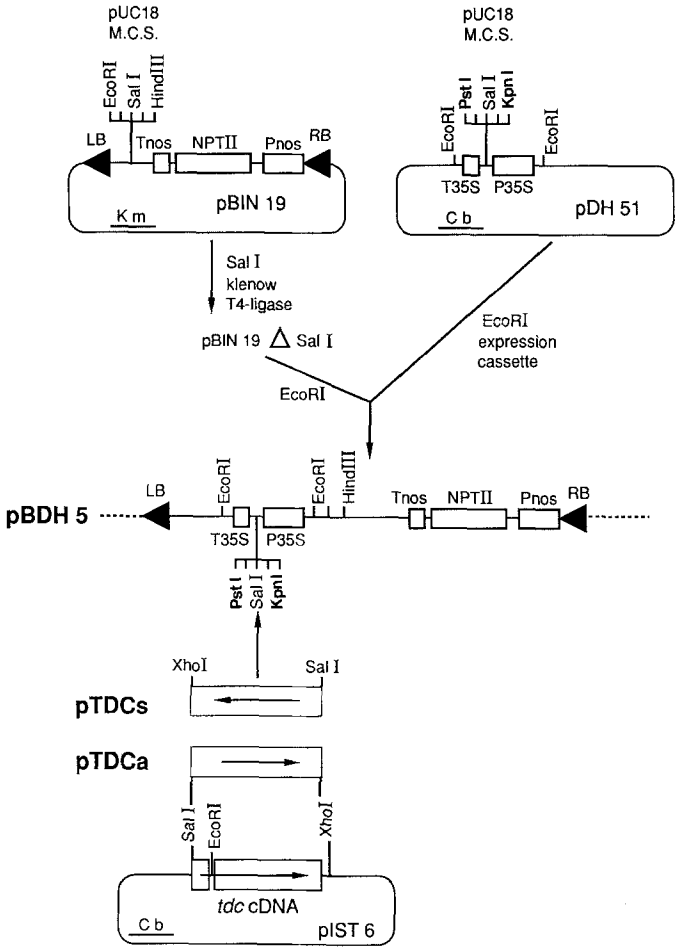

Fig. 3. Construction of the binary plant expression vector pBDH5 and generation of transcriptional fusions between an artificially completed *tdc* cDNA sequence and the CaMV 35S promoter and terminator in pBDH5. Construct pTDCs leads to overexpression of *tdc* mRNA, while pTDCa leads to expression of antisense *tdc* RNA. Abbreviations, M.C.S., multiple cloning site; LB, RB, left and right T-DNA border repeats; P35S and Pnos refer to the CaMV 35S and nopaline synthase promoter sequences; T35S and Tnos refer to the CaMV 35S and nopaline synthase terminator sequences; NPTII, neomycin phosphotransferase gene; Km, bacterial kanamycin resistance selection marker; Cb, bacterial carbenicillin resistance selection marker.

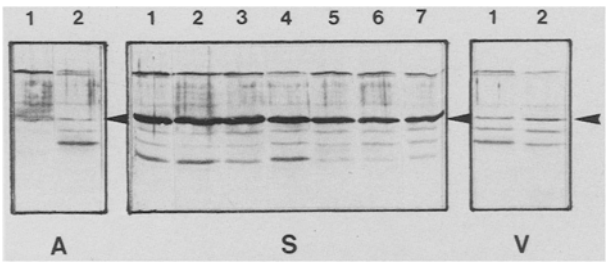

Fig. 4. Western blot analysis of *tdc* transformed crown gall tumour calluses of *C. roseus*. Panel A, S and V display pTDCa (antisense), pTDCs (sense) and pBDH5 (vector) transformed calluses. The 47 kDa TDC monomer is marked by arrowheads.

Table 1. Analysis of TDC activity, tryptamine and terpenoid indole alkaloid content in *tdc* transgenic tumour tissues of C. roseus

		TDC activity pkat per mg protein	*Tryptamine nmol per mg DW*	*Ajmalicine nmol per mg DW*	*Serpentine derivative nmol per mg DW*
Sense	1	66	3.2	ND	ND
	2	54	1.6	0.24	0.09
	3	73	1.6	ND	0.11
	4	186	5.7	ND	ND
	5	103	2.0	0.47	0.27
	6	169	3.3	0.19	0.18
	7	133	4.1	ND	ND
Antisense	1	ND	ND	ND	ND
	2	2	–	–	–
Vector	1	4	0.7	0.32	0.22
	2	16	0.9	ND	0.40

TDC activity, tryptamine content and production of ajmalicine and an unidentified serpentine derivative in *tdc* transformed *C. roseus* crown gall tumour calluses. Sense, Antisense and Vector refer to pTDCs, pTDCa and pBDH5 transformed calluses. TDC activity is given as an average value of two independent measurements. The tryptamine, ajmalicine and serpentine derivative contents were determined once for each tissue.
DW: dry weight; ND: not detectable; –: not done

pTDCa transformed calluses. The observed variation in TDC activity in the pTDCs-transformed calluses was about threefold, ranging from 54 pkat per mg protein to 186 pkat per mg of protein. These levels were at least ten times higher than those in pBDH5 control calluses.

A positive correlation was observed between TDC activity and the tryptamine content of the transformed calluses. The production of terpenoid indole alkaloids (TIAs) by the tumour calluses was monitored by HPLC analysis. Accumulation of ajmalicine and an unidentified serpentine derivative was observed only in some of the calluses. The unidentified serpentine derivative displayed a similar UV-absorption spectrum but a different retention time than serpentine. Despite substantially increased tryptamine levels, comparable amounts of ajmalicine and the serpentine derivative were detected in pTDCs and pBDH5 calluses. This indicates that other enzymes or other, as yet unknown, factors involved in TIA biosynthesis are probably limiting the production of TIAs in the transgenic tissues. Both mentioned compounds were not detected in pTDCa transformed calluses. With regard to growth rate and morphological appearance, no clear differences were noticed between the tumour calluses transformed with the T-DNAs of pTDCs, pTDCa and pBDH5.

Discussion

The enzyme tryptophan decarboxylase (TDC), which catalyses the conversion of the primary metabolite tryptophan into tryptamine, may represent a yield-limiting enzyme in the pathway of secondary metabolism in *C. roseus* leading to formation of terpenoid indole alkaloids. The accumulation characteristics of *tdc* mRNA in several *C. roseus* tissues indicate extensive regulation of *tdc* expression. The low *tdc* mRNA levels in cell suspension and callus cultures versus the at least tenfold higher levels in roots suggest that *tdc* expression may be incompatible with active cell proliferation. Such incompatibility would explain the absence or very much reduced production of TIAs in the rapidly dividing phytohormone independent oncogenic *C. roseus* tissues. Genetic manipulation of the *tdc* expression level might bypass one of the regulatory controls on TIA biosynthesis.

Amino acid sequence analysis of purified TDC monomers, isolated from induced *C. roseus* cell cultures, revealed that 13 N-terminal amino acids coded for by *tdc* mRNA were missing, suggesting a processing of the protein. As the TDC protein has been shown to reside in the cytosol (De Luca *et al.*, 1987; Stevens *et al.*, 1993), it seems unlikely that these N-terminal amino acids function as a signal peptide necessary for correct translocation of the enzyme. Furthermore, according to the rules of von Heijne (1986), the sequence of this peptide itself does not indicate such a role. A construct was generated leading to a completed *tdc* cDNA clone in which the ATG on position +31 serves as translation start. This construct resulted in TDC activities ranging from 54–186 pkat per mg of protein. Songstad *et al.* (1990) reported on transformed tabacco plants expressing a full-length *tdc* cDNA clone under control of the CaMV

35S promoter and nopaline synthase terminator. In leaf tissue of these transgenic plants, TDC activities were detected ranging from 6.8–30.2 pkat per mg of protein. We also generated transgenic *N. tabacum* plants expressing our slightly modified *tdc* cDNA. In green tissues, TDC activities were detected ranging from 4.2–69.2 pkat per mg of protein (Goddijn *et al.*, 1993). These data are comparable to those obtained by Songstad *et al.*, suggesting that the activity of the TDC protein is not affected by altering its N-terminal amino acid sequence. The higher TDC activities measured in transgenic *C. roseus* calluses compared to transgenic tobacco plants are difficult to explain. Factors related to the plant species as well as type of tissue in which *tdc* cDNA is overexpressed may contribute to the observed difference.

Overexpression of *tdc* cDNA in *C. roseus* tumour calluses resulted in the accumulation of tryptamine. Tryptamine levels as high as 5.7 nmol per mg of dry weight were measured which is approximately six times higher than the tryptamine levels in control calluses. The increased tryptamine levels, however, did not result in stimulation of TIA production, as indicated by the unaltered levels of ajmalicine and a serpentine derivative. This implies that steps in TIA biosynthesis other than conversion of tryptophan into tryptamine by TDC must be yield-limiting in the *tdc* cDNA transformed tumour calluses. Several mutually non-exclusive limitations might explain the unaltered TIA concentrations. The excess of tryptamine formed may not be available for condensation with secologanin by the enzyme strictosidine synthase when both components are located in different cellular compartments in the cell. Alternatively, secologanin or strictosidine synthase activity may be scarce or lacking. Furthermore, insufficient activity levels of one or all of the enzymes involved in processing of strictosidine into various TIAs may pose barriers to production.

Regardless of the yield-limiting step(s) involved, one should also consider the tumorous state of the *tdc* cDNA-transgenic *C. roseus* calluses studied, which is caused by the auxin and cytokinin biosynthetic genes of *A. tumefaciens* T_L-DNA (Hooykaas and Schilperoort, 1992). Investigations in our laboratory demonstrated that the genes coding for tryptophan decarboxylase and strictosidine synthase are coordinately expressed and repressed at the transcriptional level by the auxins IAA, NAA and 2,4-D (Goddijn *et al.*, 1992; Pasquali *et al.*, 1992). It is conceivable therefore that the auxin biosynthetic genes suppress TIA production in tumour calluses overexpressing *tdc* cDNA by inhibiting expression of other TIA biosynthetic genes. That the tumorous state of *C. roseus* calluses may hinder expression of TIA biosynthetic genes is evidenced by the observation in our laboratory (R.J. de Kam, unpublished results) that accumulation of strictosidine synthase mRNA in *C. roseus* tumor calli parallels the poor accumulation of endogenously encoded *tdc* mRNA. It would be interesting therefore to examine the effects of *tdc* transgenic cDNA overexpression on TIA production in non-tumorous *C. roseus* calluses. Such studies are currently in progress.

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