Plant Cell Reports (1997) 17: 50–54

D. Hallard · R. van der Heijden · R. Verpoorte
M. I. Lopes Cardoso · G. Pasquali · J. Memelink
J. H. C. Hoge

Suspension cultured transgenic cells of *Nicotiana tabacum* expressing tryptophan decarboxylase and strictosidine synthase cDNAs from *Catharanthus roseus* produce strictosidine upon secologanin feeding

Received: 25 February 1996 / Revision received: 16 August 1996 / Accepted: 30 September 1996

Abstract A transgenic cell suspension culture of *Nicotiana tabacum* L. 'Petit Havana' SR1 was established expressing tryptophan decarboxylase and strictosidine synthase cDNA clones from *Catharanthus roseus* (L.) G. Don under the direction of cauliflower mosaic virus 35S promoter and nopaline synthase terminator sequences. During a growth cycle, the transgenic tobacco cells showed relatively constant tryptophan decarboxylase activity and an about two- to sixfold higher strictosidine synthase activity, enzyme activities not detectable in untransformed tobacco cells. The transgenic culture accumulated tryptamine and produced strictosidine upon feeding of secologanin, demonstrating the in vivo functionality of the two transgene-encoded enzymes. The accumulation of strictosidine, which occurred predominantly in the medium, could be enhanced by feeding both secologanin and tryptamine. No strictosidine synthase activity was detected in the medium, indicating the involvement of secologanin uptake and strictosidine release by the cells.

Key words *Nicotiana tabacum* · Secologanin · Terpenoid indole alkaloids · Strictosidine synthase · Tryptophan decarboxylase

Abbreviations *KIN* Kinetin · *LS* Linsmaier and Skoog medium · *NAA* 1-Naphtaleneacetic acid · *STR* Strictosidine synthase · *TDC* Tryptophan decarboxylase

Communicated by W. Barz

D. Hallard · R. van der Heijden (✉) · R. Verpoorte
Division of Pharmacognosy, Leiden/
Amsterdam Center for Drug Research, Leiden University,
Gorlaeus Laboratories, PO Box 9502, 2300 RA Leiden,
The Netherlands
Fax no.: +31-71-527-45-11
e-mail: heijden@chem.leidenuniv.nl

M. I. Lopes Cardoso · G. Pasquali · J. Memelink · J. H. C. Hoge
Institute of Molecular Plant Sciences, Leiden University,
Clusius Laboratory, PO Box 9505, 2300 RA Leiden,
The Netherlands

Introduction

Plant secondary metabolism gives rise to the formation of a vast array of chemically complex compounds, many of which are commercially important. Initial studies on plant secondary metabolism focused on the chemistry of the metabolites and their biosynthesis. Subsequently, research efforts partly shifted to the characterisation of the biosynthetic enzymes involved. Nowadays, isolation of the genes coding for these enzymes and elucidation of the regulatory mechanisms which control their expression are also central issues in the field of plant secondary metabolism. Once cloned, such genes can be exploited to produce the corresponding enzymes or for metabolic engineering. Examples of these options are the production of the enzyme strictosidine synthase (STR, EC 4.3.3.2) in transgenic insect cells expressing a *str* cDNA clone from *Rauvolfia serpentina* (Kutchan et al. 1991), and accumulation of tryptamine in transgenic tobacco plants due to the expression of a cDNA clone from *Catharanthus roseus* coding for the enzyme tryptophan decarboxylase (TDC, EC 4.1.1.28) (Songstad et al. 1990, 1991; Goddijn et al. 1993; Poulsen et al. 1994). A *tdc* cDNA clone from *C. roseus* was also expressed in transgenic canola plants, modifying the glucosinolate content (Chavadej et al. 1994), and in transgenic *Peganum harmala* cells resulting in a tenfold increase in serotonin production (Berlin et al. 1993). Other examples of genetic engineering of plant secondary metabolism have been recently reviewed (Nessler 1994).

Rapid advances in cDNA cloning of genes encoding enzymes in plant secondary metabolism provide an ever-increasing range of opportunities for exploring the possibilities of metabolic engineering in plants and derived organ, tissue and cell culture systems. In our laboratories, we have isolated cDNA clones encoding the enzymes TDC and STR from *C. roseus* (Goddijn 1992; Pasquali et al. 1992), a model plant for studies on plant secondary metabolism, in particular the biosynthesis of terpenoid indole alkaloids (Fig. 1).

As a first step in transferring combinations of terpenoid indole alkaloid biosynthetic genes into the cells of

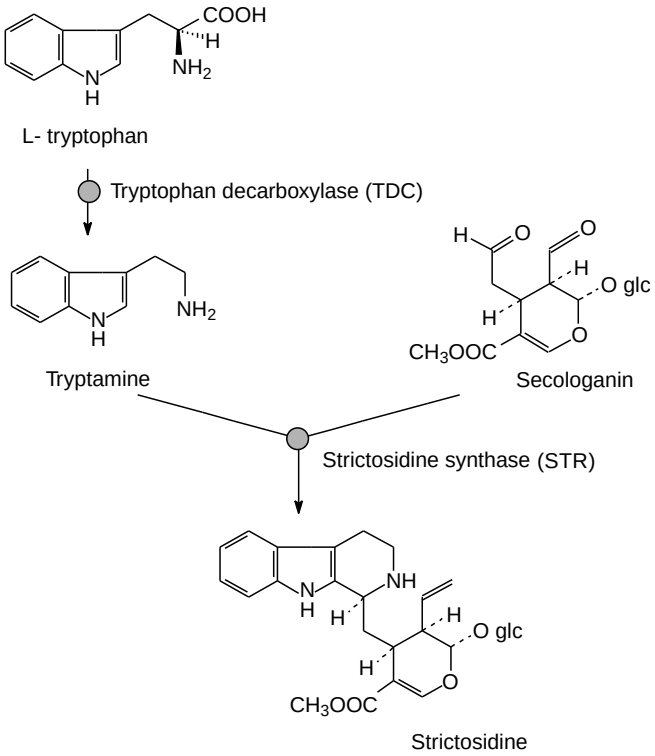


Fig. 1 Tryptophan decarboxylase (*TDC*) and strictosidine synthase (STR) in the biosynthesis of strictosidine, the general precursor of terpenoid indole alkaloids

plants that do not possess such genes, we report here the functional expression of both *tdc* and *str* gene constructs in tobacco cells. After feeding of secologanin, these cells produce strictosidine, which is predominantly released into the culture medium.

Materials and methods

Construction of binary vector for plant transformation

Full-length *tdc* and *str* cDNA clones from *C. roseus* (G. Pasquali and J. Memelink, unpublished data) were fused in sense orientation to a CaMV 35S promoter with double enhancer and the *nos* terminator using the expression cassette plasmids pMOG183 and pMOG463 (Mogen International, Leiden, The Netherlands), respectively. These constitutively expressing versions of the *C. roseus tdc* and *str* genes were subsequently inserted, together with a β-glucuronidase intron (*gus* int) reporter gene (Vancanneyt et al. 1990), in the *nptII*-selection-gene-containing T-DNA region of the binary plant vector pMOG402 (Mogen International). A schematic representation of the T-DNA region of the resulting binary vector construct is depicted in Fig. 2. Construction details are available on request. The binary vector was introduced by triparental mating (Ditta et al. 1980) into *Agrobacterium tumefaciens* strain LBA4404 for transformation of tobacco.

Fig. 2 T-DNA region of binary vector construct used for *Agrobacterium tumefaciens*-mediated transformation of *Nicotiana tabacum* (*LB* left border, *RB* right border, *35S pr* promoter sequences from CaMV 35S promoter, *35S tr* terminator sequences from CaMV 35S terminator, *NOS pr* promoter sequences from nopaline synthase promoter, *NOS tr* terminator sequences from nopaline synthase terminator, *NPTII* neomycin phosphotransferase coding region, *TDC* tryptophan decarboxylase coding region, *STR* strictosidine synthase coding region, *GUS-Int* intron-interrupted coding region of β-glucuronidase)

Tobacco leaf disk transformation and establishment of transgenic cell suspension culture

Nicotiana tabacum cultivar Petit Havanna SR1 plants were grown axenically on solidified MS medium (Murashige and Skoog 1962) containing 30 g/l sucrose at 25°C with a photoperiod of 12 h. Leaf disk transformation with *A. tumefaciens* strain LBA4404 harbouring the described binary vector construct was performed essentially as described by Horsch et al. (1985). Transgenic plants were maintained on solidified MS medium containing 100 mg/l cefotaxim, 100 mg/l vancomycin and 100 mg/l kanamycin. Leaves of one of the transgenic plants were excised and placed on agar-solidified LS medium (Linsmaier and Skoog 1965) containing 2.0 mg/l 1-naphthaleneacetic acid (NAA), 0.2 mg/l kinetin (KIN) and 30 g/l sucrose to produce callus. Callus was excised, grown for 1 week on the same solid medium, and then transferred to liquid LS medium containing 2.0 mg/l NAA, 0.2 mg/l KIN and 30 g/l sucrose to obtain a cell suspension culture. The resulting culture was grown at 25±1°C under dim light on a gyratory shaker at 100 rpm, and subcultured weekly.

Characterisation of the transgenic tobacco culture

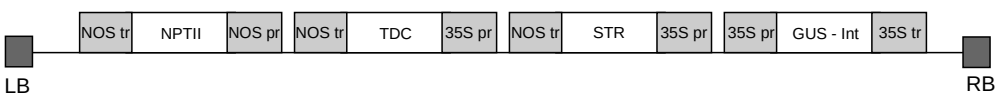
Erlenmeyer flasks of 250 ml, containing 50 ml of culture medium, were inoculated with 5±0.1 g cells from a 7-day-old culture of transgenic tobacco cells. The cells were harvested 2, 5, 7 and 10 days after inoculation. After harvesting, the cells were immediately frozen in liquid nitrogen and stored at –80°C.

For feeding experiments, filtration-sterilised aqueous solutions of secologanin and tryptamine were added to the cells 5 days after culture inoculation. Four feeding conditions were used: A, controls, feeding of an equivalent volume of sterile distilled water; B, feeding of tryptamine (final concentration 0.5 mM); C, feeding of secologanin (final concentration 0.5 mM); D, feeding of both tryptamine and secologanin (final concentration 0.5 mM each). The cultures were harvested 1, 3 and 5 days after feeding. The culture medium was separated from the biomass by filtration. The cells were immediately frozen in liquid nitrogen and stored at –80°C. The media were centrifuged at 5,000 *g* for 20 min. The supernatants were frozen in liquid nitrogen and stored at –80°C. Experiments were performed in duplicate.

For the feeding conditions A and D, an untransformed tobacco cell culture was used to measure non-transgene-encoded formation of strictosidine.

Enzyme assays

Protein extracts were prepared by homogenising the frozen cells in a Waring blender cooled with liquid nitrogen. The powdered cells were thawed in the presence of polyvinylpolypyrrolidone (50 mg/g fresh weight) and 0.1 M sodium phosphate buffer pH 7, containing



2 mM EDTA and 4 mM dithiothreitol (1 ml/g fresh weight). The extracts were clarified by centrifugation (10,000 *g*, 30 min, 4°C). For desalting, the supernatants were applied on a Sephadex G-25 (PD10, Pharmacia) column and eluted with 0.1 M sodium phosphate buffer, pH 7, containing 2 mM EDTA (buffer A). TDC and STR activities were assayed in triplicate according to Pennings et al. (1987, 1989). Protein concentrations were assayed in triplicate by the method described by Peterson (1977) with bovine serum albumin as standard.

Determination of tryptamine, secologanin and alkaloids

Tryptamine contents during a growth cycle of the transgenic tobacco cell suspension were determined by HPLC, by injection of TDC enzyme assay blanks made on crude non-desalted enzyme extracts. For the determination of tryptamine concentrations in cells and media from the feeding experiments, freeze-dried cells or culture media were extracted according to Schripsema and Verpoorte (1992) and analysed by means of HPLC (van der Heijden et al. 1987) equipped with photodiode-array detection. Strictosidine was assayed according to Moreno et al. (1993). For the determination of secologanin, aliquots of protein extracts (before desalting, see above) were lyophilised, as were the culture media. The residues were dissolved in buffer A. Secologanin was quantified in duplicate, as described elsewhere (D. Hallard et al., in preparation). This assay is based on the condensation of secologanin with tryptamine, yielding strictosidine, in a reaction catalysed by STR. Subsequently, the formation of strictosidine is quantified by HPLC.

Results and discussion

Establishment and characterisation of the transgenic tobacco cell suspension culture

The binary vector T-DNA region depicted in Fig. 2 was transferred into tobacco plants by leaf disk transformation with *A. tumefaciens*. Northern and Western blot analysis as well as enzyme assays confirmed the transgenic status of the obtained plants (Lopes Cardoso 1996). The leaves of one of these plants were subsequently used to establish a transgenic cell suspension culture. Before experimentation, the resulting culture, light-green in colour, was allowed to stabilise for 10 months.

The transgenic tobacco cell suspension culture was characterised for growth, TDC and STR activities and accumulation of tryptamine (Table 1). During a growth cycle, the culture displayed a relatively constant TDC activity of 12–26 pkat/mg protein. Due to this activity, tryptamine accumulated in the cells at levels fluctuating between 6.4–12.6 μg/g fresh weight. STR activity in the transgenic tobacco cells was about two- to sixfold higher than TDC activity, reaching a peak of 104 pkat/mg protein at day 10 of the growth cycle. Tryptamine accumulation and TDC as well as STR enzyme activities were undetectable in untransformed tobacco cell suspensions from our cell culture collection (data not shown).

Table 1 *TDC* and *STR* activities and accumulation of tryptamine in the biomass of suspension-cultured transgenic tobacco cells expressing *Catharanthus roseus tdc* and *str* cDNA clones. Values are mean, $n=2$ {*GI* (growth index)=[FW(*t*)–FW(0)]/FW(0), where *FW*=fresh weight}

Time (days)	GI	Tryptamine (μg/g FW)	Specific activities (pkat/mg protein)	
			TDC	STR
2	0.18	11.8	26	49
5	1.21	6.4	25	44
7	3.66	8.1	12	28
10	4.27	12.6	16	104

In *C. roseus* suspension cultures, TDC activity usually peaks during the first days after subculture and then declines rapidly. In *P. harmala* suspension cultures also, endogenous TDC activity peaks sharply during the first 24–48 h after transfer to fresh medium. However, in transgenic suspension cultures of *P. harmala* expressing *C. roseus* cDNA coding for TDC, TDC activity remained elevated for at least 5 days after subculture (Berlin et al. 1993). This observation and that reported here for transgenic tobacco cells indicate that the CaMV 35S promoter enables high and stable activity of a rate-limiting secondary metabolite biosynthetic enzyme in heterologous systems.

The accumulation of tryptamine in tobacco cells did not appear to have any toxic effects, judging by the rapid accumulation of biomass during suspension culture (Table 1). The levels of tryptamine found in the transgenic suspension-cultured cells (about 10 μg/g fresh weight) are similar to those found in leaves of *tdc*, or *tdc* and *str*, transgenic tobacco plants (ranging from 18 to 66 μg/g fresh weight; Lopes Cardoso 1996) and in *C. roseus* cell suspension cultures (about 12 μg/g fresh weight; D. Hallard, unpublished data).

Feeding of secologanin to the transgenic tobacco culture

In previous investigations it was shown that tryptamine, accumulating in *C. roseus* suspension-cultured cells, is available for terpenoid indole alkaloid biosynthesis: upon feeding of secologanin to the cultures, strictosidine is formed (Moreno et al. 1993). Here, similar feeding experiments were performed with the transgenic tobacco cells, to find out if secologanin can reach the compartment(s) where tryptamine and STR are located within the plant cells. Moreover, such experiments may demonstrate the in vivo functionality of transgenically produced TDC and STR enzymes. Addition of secologanin to the transgenic tobacco culture indeed resulted in the accumulation of strictosidine (Fig. 3). Some of the strictosidine formed by the transgenic cells was recovered from the medium, but strictosidine concentrations rapidly decreased. Secologanin disappeared more quickly, and to a larger extent from the medium, than it was incorporated into strictosidine. Similar mechanisms may perhaps be involved in the degradation of secologanin and strictosidine. Strictosidine (and secologanin) were stable for at least 40 h in fresh culture medium, when incubated under identical culture conditions. Furthermore, in medium separated from the transgenic tobacco culture after 5 days of growth, secologanin

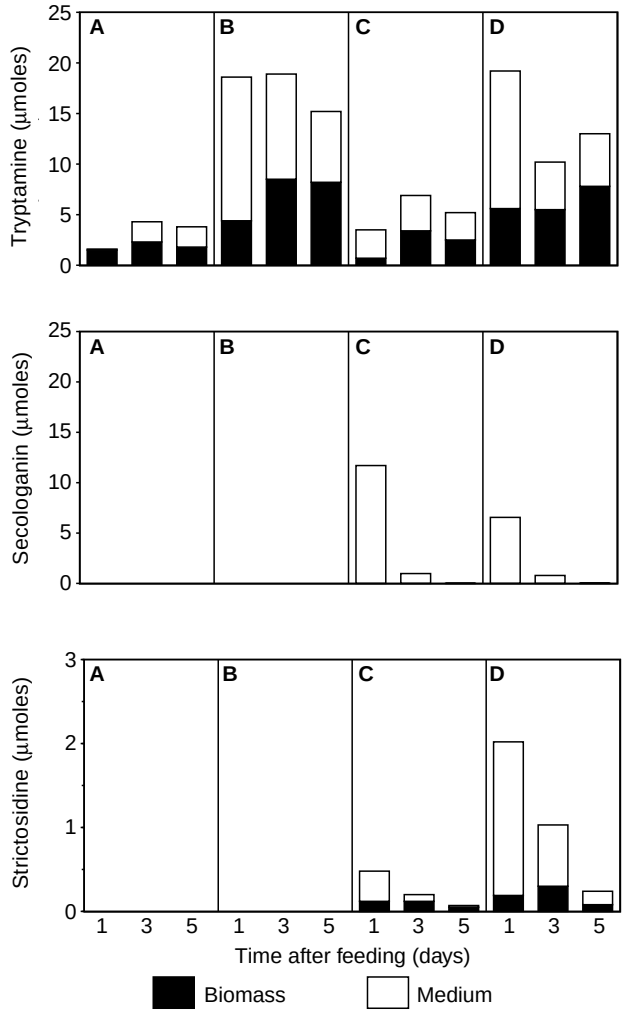


Fig. 3 Amount of tryptamine, secologanin and strictosidine in the biomass and medium of suspension cultures of transgenic tobacco cells expressing *Catharanthus roseus* TDC and SSS genes. At day 0, the cultures were fed with 25 µmol of tryptamine (*B*), 25 µmol secologanin (*C*) or tryptamine and secologanin (25 µmol each, *D*). Control cultures (*A*) received an equal volume of sterile water

and strictosidine did not decompose (data not shown). Thus, the disappearance of these iridoid-glucosides in the feeding experiment must be mediated by the transgenic tobacco cells. Analysis of media and biomass by HPLC-UV/VIS confirmed the identity of strictosidine by retention time and its UV spectrum, but no degradation products of strictosidine could be detected. Furthermore, neither TDC nor STR activities were detected in the culture media, suggesting that strictosidine was produced inside the cells. This would have required uptake of secologanin into the cells, where it was converted to strictosidine, as no secologanin could be detected inside the cells. STR has been shown to be localised in the vacuoles of *C. roseus* (McKnight et al. 1991; Stevens et al. 1993) and transgenic tobacco cells (McKnight et al. 1991). In the gene construct we used for transformation of the tobacco cells, the sequence coding for the signal peptide of STR was also present. Vacuolar localisation of STR is therefore expected. The conversion of tryptophan into tryptamine by TDC has been shown to occur in the cytosol of *C. roseus* cells (De Luca and Cutler 1987; Stevens et al. 1993). Upon its formation, tryptamine is thought to accumulate in the vacuole. Similar processes could also occur in the transgenic tobacco cells; however, it has not yet been demonstrated that secologanin is able to enter the vacuoles of transgenic tobacco cells.

The accumulation of strictosidine was enhanced about fourfold when both secologanin and tryptamine were fed to the transgenic cell culture (Fig. 3). Feeding of both compounds to an untransformed tobacco cell culture did not give rise to strictosidine accumulation in the medium or biomass (data not shown), thereby excluding their conversion into strictosidine by mechanisms other than transgene-encoded enzyme activity. Maximum transgene-encoded incorporation of secologanin into strictosidine was about 8%. Feeding of secologanin and/or tryptamine to the suspension of transgenic tobacco cells did not affect the final dry weight yield, although secologanin initially caused slight inhibitory effects (data not shown).

It is concluded from these experiments that gene constructs encoding TDC and STR of *C. roseus* can be expressed in tobacco cells in such a way that the corresponding enzyme activities are available for, and cooperative in, the biosynthesis of a secondary metabolite which is foreign to tobacco. These gene constructs are currently being used for transformation of various plant species known for their accumulation of secologanin. The obtained transgenic cultures may thus accumulate strictosidine as a foreign secondary metabolite. In addition, the transgenic tobacco cells described here serve in our laboratories as a source of STR, which can be used in an assay to determine secologanin contents in plant tissues (D. Hallard et al., in preparation).

Acknowledgements We wish to thank Dr. S. R. Jensen (Lyngby, Denmark) for his kind gift of secologanin. D. H. wishes to express his gratitude to the EC for a COMETT grant and appreciates the linguistic assistance of Pauline de Graaf. The research of R. vd H. has been made possible by a fellowship of the Royal Netherlands Academy of Arts and Sciences. M. I. L. C. and G. P. are indebted to JNICT, Portugal and CNPq, Brazil, respectively, for PhD fellowships.

References

Berlin J, Rügenhagen C, Dietze P, Fecker LF, Goddijn OJM, Hoge JHC (1993) Increased production of serotonin by suspension and root cultures of *Peganum harmala* transformed with a tryptophan decarboxylase cDNA clone from *Catharanthus roseus*. Transgenic Res 2:336–344

Chavadej S, Brisson N, McNeil JN, De Luca V (1994) Redirection of tryptophan leads to production of low indole glucosinolate canola. Proc Natl Acad Sci USA 91:2166–2170

De Luca V, Cutler AJ (1987) Subcellular localization of enzymes involved in indole alkaloid biosynthesis in *Catharanthus roseus*. Plant Physiol 85:1099–1102

Ditta G, Stanfield S, Corbin D, Helinski DR (1980) Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc Natl Acad Sci USA 77:7347–7351

Goddijn OJM (1992) Regulation of terpenoid indole alkaloid biosynthesis in *Catharanthus roseus*: the tryptophan decarboxylase gene. PhD thesis, Leiden University

Goddijn OJM, Duijn Schouten PM van der, Schilperoort RA, Hoge JHC (1993) A chimaeric tryptophan decarboxylase gene as a novel selectable marker in plant cells. Plant Mol Biol 22:907–912

Heijden R van der, Lamping PJ, Out PP, Wijnsma R, Verpoorte R (1987) High-performance liquid chromatographic determination of indole alkaloids in a suspension culture of *Tabernaemontana divaricata*. J Chromatogr 396:286–295

Horsch RB, Fry JE, Hoffmann NL, Eichhioltz Rogers SG, Fraley RT (1985) A simple and general method of transferring genes into plants. Science 227:1229–1231

Kutchan TM, Dittrich H, Bracher D, Zenk MH (1991) Enzymology and molecular biology of alkaloid biosynthesis. Tetrahedron 47:5945–5954

Linsmaier EM, Skoog F (1965) Organic growth factor requirements of tobacco tissue cultures. Physiol Plant 18:100–127

Lopes Cardoso MI (1996) Analysis and modification of the expression of genes involved in plant secondary metabolism: genes encoding early steps in terpenoid indole alkaloid biosynthesis in *Catharanthus roseus*. PhD thesis, Catholic University of Portugal

McKnight TD, Bergey DR, Burnett RJ, Nessler CL (1991) Expression of enzymatically active and correctly targeted strictosidine synthase in transgenic tobacco plants. Planta 185:148–152

Moreno PRH, Heijden R van der, Verpoorte R (1993) Effect of terpenoid precursor feeding and elicitation on formation of indole alkaloids in cell suspension cultures of *Catharanthus roseus*. Plant Cell Rep 12:702–705

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473–497

Nessler CL (1994) Metabolic engineering of plant secondary products. Transgen Res 3:109–115

Pasquali G, Goddijn OJM, Waal A de, Verpoorte R, Schilperoort RA, Hoge JHC, Memelink J (1992) Coordinated regulation of two indole alkaloid biosynthetic genes from *Catharanthus roseus* by auxin and elicitors. Plant Mol Biol 18:1121–1131

Pennings EJM, Hegger I, Heijden R van der, Duine JA, Verpoorte R (1987) Assay of tryptophan decarboxylase from *Catharanthus roseus* plant cell cultures by high-performance liquid chromatography. Anal Biochem 165:133–136

Pennings EJM, Bosch RA van den, Heijden R van der, Stevens LH, Duine JA, Verpoorte R (1989) Assay of strictosidine synthase from plant cell cultures by high-performance liquid chromatography. Anal Biochem 176:412–415

Peterson GL (1977) A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem 83:346–356

Poulsen C, Goddijn OJM, Hoge JHC, Verpoorte R (1994) Anthranilate synthase and chorismate mutase activities in transgenic tobacco plants overexpressing tryptophan decarboxylase from *Catharanthus roseus*. Transgen Res 3:43–49

Schripsema J, Verpoorte R (1992) Search for factors involved in indole alkaloid production in cell suspension cultures of *Tabernaemontana divaricata*. Planta Med 58:245–249

Songstad DD, De Luca V, Brisson N, Kurz WGW, Nessler CL (1990) High levels of tryptamine accumulation in transgenic tobacco expressing tryptophan decarboxylase. Plant Physiol 94:1410–1413

Songstad DD, Kurz WGW, Nessler CL (1991) Tyramine accumulation in *Nicotiana tabacum* transformed with a chimeric tryptophan decarboxylase gene. Phytochemistry 30:3245–3246

Stevens LH, Blom TJM, Verpoorte R (1993) Subcellular localization of tryptophan decarboxylase, strictosidine synthase and strictosidine β-D-glucosidase in suspension cultured cells of *Catharanthus roseus* and *Tabernaemontana divaricata*. Plant Cell Rep 12:573–576

Vancanneyt G, Schmidt R, O'Connor-Sanchez A, Wilmitzer L, Rocha-Sosa M (1990) Construction of an intron-containing marker gene: splicing of the intron in transgenic plants and its use in monitoring early events. Mol Gen Genet 220:245–250