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Effect of differentiation on the regulation of indole alkaloid production in *Catharanthus roseus* hairy roots

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Abstract In vitro cultures of hairy root derived from *Catharanthus roseus* accumulate higher levels of indole alkaloids than cell suspension cultures. Hairy roots were interconverted to undifferentiated cells by manipulation of the culture medium. When the concentration of micronutrients in the culture medium was five times that of Phillips and Collins (1979) medium, cell suspensions formed from the hairy roots. The alkaloid content was five times lower in the cell suspensions than in the control, but upon regeneration of the roots the alkaloid content regained its original level. The formation of cell suspensions from hairy roots was also accompanied by a reduction in tryptophan decarboxylase and the strictosidine synthase activity to less than 5% and 30%, respectively. 3-Hydroxymethylglutaryl coenzyme A reductase activity was the same in the cell suspension and in the regenerated line.

Key words *Catharanthus roseus* · Differentiation · Hairy roots · Tryptophan decarboxylase · Strictosidine synthase · 3-hydroxymethylglutaryl coenzyme A reductase

Abbreviations *TDC* Tryptophan decarboxylase · *SS* Strictosidine synthase · *HMGR* 3-hydroxymethylglutaryl coenzyme A reductase · *PC* Phillips and Collins (1979) medium · *PMSF* (phenylmethylsulfonyl fluoride)

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Introduction

During the last decade, the effect of cell organisation on the formation and accumulation of secondary metabolites in plant cell and tissue cultures has been well documented (Lindsey and Yeoman 1983; Flores 1987). In general, it has been recognised that a cell culture which does not accumulate secondary metabolites will do so when induced to differentiate into tissue/organs. Some examples are the occurrence of tropane alkaloids in roots differentiated from callus of *Atropa belladona* and *Hyoscyamus muticus* (Collinge and Yeoman 1986), the production of morphinane alkaloids during somatic embryogenesis of *Papaver somniferum* (Galewsky and Nessler 1986), the synthesis of indole alkaloids in root and shoot cultures of *Catharanthus roseus* (Endo et al. 1987) and the accumulation of cardenolides during *Digitalis lanata* somatic embryogenesis (Greidziak et al. 1990).

Hyoscyamine has been shown to only accumulate in organised systems, such as hairy roots, and is present only in trace amounts; it is completely absent in undifferentiated systems such as callus cultures (Flores 1987). Transformed roots of *C. roseus* accumulate indole alkaloids by least one order of magnitude more than do suspension cultures (Ciau-Uitz et al. 1994).

An excellent model to use when studying the role of differentiation in the biosynthesis of secondary metabolites is that of undifferentiated-redifferentiated hairy root cultures. Repunte et al. (1993) reported that cell aggregates derived from hairy roots of horseradish were capable of regenerating hairy roots. Peroxidase activity in regenerated lines was comparable to that of the original hairy roots. Also, *Hyoscyamus muticus* hairy root cultures produced calli upon transfer to medium containing growth regulators. These unorganised calli produced only trace amounts of alkaloids, but upon their return to hormone-free medium they recovered a stable hairy root phenotype with the full content of alkaloids (Flores 1987). Likewise, in the presence of hormones, hairy root cultures of *C. roseus* have been found to form suspension

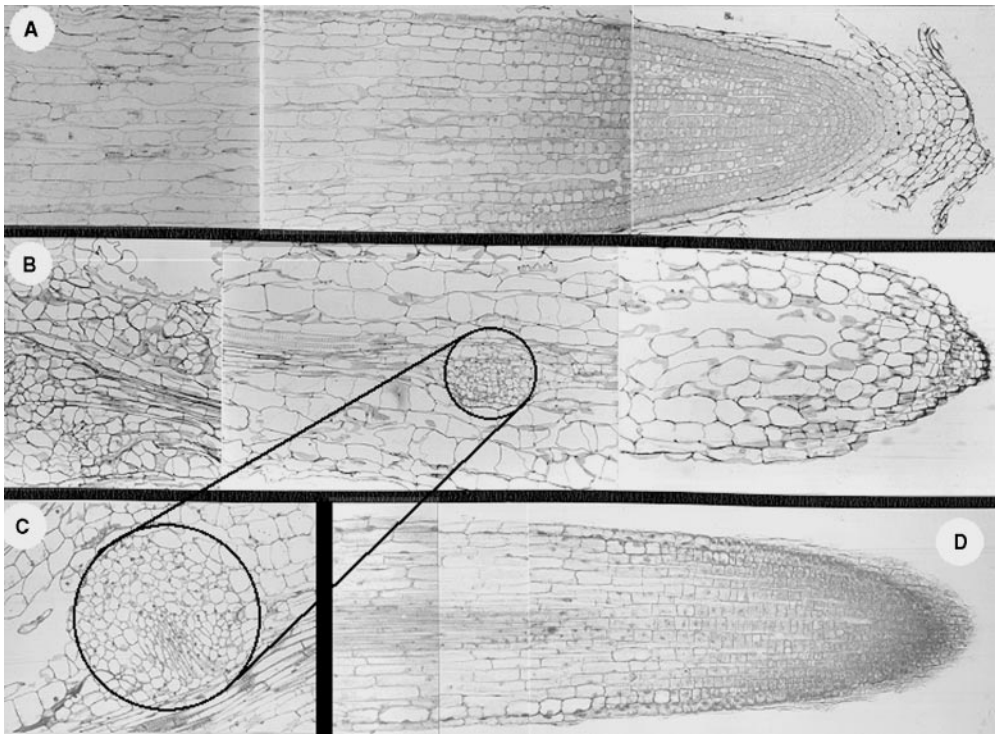

Fig. 1A–D Morphological changes present in the roots of *Catharanthus roseus* through the process of dedifferentiation. **A** Control line J2, **B** hairy roots after 24 days in the dedifferentiation medium, **C** close-up of the meristematic zone, where a lateral root is being formed, which originated all dedifferentiated cells, **D** regenerated line R2

cultures, which partially loose the ability to produce catharanthine. The ability to produce catharanthine is recovered upon the transfer of such cells to the initial medium and in step with the recovery of the hairy root phenotype (Jung et al. 1995).

Here, we describe experiments that focused on the activities of enzymes involved in the biosynthesis and accumulation of monoterpene indole alkaloids as regulated by the grade of differentiation in *C. roseus* hairy root cultures.

Materials and methods

Plant material

Transformed roots were obtained through genetic transformation of seedling roots of *Catharanthus roseus* (L.) G. Don with *Agrobacterium rhizogenes* strain 1855 bearing plasmid pBI 121.1 (Ciau-Uitz et al. 1994). Hairy roots were maintained through subculturing every 21 days using half-strength Gamborg B5 medium (B5/2; Gamborg et al. 1968) supplemented with 3% of sucrose. The root cultures were kept on orbital shakers at 100 rpm and 25 °C in the dark (Ciau-Uitz et al. 1994) .

Root differentiation-dedifferentiation

Dedifferentiated cell suspension cultures were obtained from transformed roots using Phillips and Collins (1979) liquid medium without phytohormones and with five times the concentration of micronutrients. Twenty-six days of culture led to formation of a cell suspension. This cell suspension was subcultured in the same medium. To redifferentiate cells to roots, we transferred the cell suspension to solid B5 medium at half the ionic strength. The new roots were isolated and subcultured under the same conditions as the original roots (control).

Alkaloid determination

Total alkaloids were extracted as described in Monforte-González et al. (1992), and their total content was determined spectrophotometrically at 280 nm. Ajmalicine was identified by thin-layer chromatography in a solvent system of chloroform : acetone (8 : 2) and was scanned afterwards in a Shimadzu scanner for quantification (Monforte-González et al. 1992).

HPLC alkaloid analysis

HPLC analyses were carried out at room temperature at a flow rate of 1.5 ml/min on a 4.6-mm (d)×250-mm Ultrasphere ODS, 4-μm particle size column (Beckman). The mobile phase consisted of acetonitrile: 0.01 *M* $(NH_4)_2HPO_4$ (43 : 57). The solution was filtered through a 22-μm nylon filter and degassed under vacuum.

Enzyme activity assays

The tryptophan decarboxylase (TDC) assay was performed as described in Islas et al. (1994). The strictosidine synthase (SS) activity assay activity was assayed as described in Pennings et al. (1989).

3-Hydroxy-3-methyl glutaryl coenzyme A reductase (HMGR) activity was measured according to Chappell and Nable (1987) with the following modifications. The extract was prepared by homogenising transformed roots in 2 volumes of 100 m*M* K-phosphate buffer (pH 7.5), 250 m*M* sucrose, 50 m*M* KCl, 20 m*M* EDTA and, 1 m*M* β-mercaptoethanol and then filtering the suspension through cheesecloth. The filtrate was centrifuged T 1,000 *g* for 15 min; the supernatant was subsequently centrifuged for 45 min at 100,000 *g*. The pellet was resuspended in a buffer containing 100 m*M* phophate, pH 7.5, 50 m*M* DTT, 5 m*M* EDTA, 20% glycerol, 1 m*M* PMFS and 7.5 μg/ml of leupentine. The entire extraction was carried out at 4 °C.

The HMGR activity assay measures the conversion of ^{14}C-HMG-CoA to ^{14}C-mevalonte (MVA). The assay was performed in a final volume of 26 μl and was initiated with the addition of 0.66 nmol of C-HMG-CoA (0.33 mol/ml; 221.63 Bq/ml to the reaction mixture containing 5–50 μg protein, 5 m*M* DTT, 3m*M* β-NADPH and

100 m*M* K-phosphate buffer, pH 7.5. After incubation at 30 °C for 5 min, the reaction was terminated with 5 µl of 6 *N* HCl and 100 mg/ml mevalonte lactone. The ^{14}C-MVA was first allowed to lactonise for 15 min at 18 °C; subsequently, 125 µl K-phosphate buffer and 300 µl ethyl acetate were added to the lactonized ^{14}C-MVA, and the mixture was vortexed for 3 min and centrifugerd for 2 min in a microfuge at full velocity. An aliquot of 150 µl of the upper phase was taken and added to a scintillation vial containing 5 ml of scintillation liquid, and the radioactivity was determined.

The protein content of the enzyme was determined as described by Peterson (1977), with albumin as a standard.

Results and discussion

The strategy used here to dedifferentiate hairy roots of *C. roseus* was to modify the micronutrient levels of the Phillips and Collins (1975) medium. Twenty-six days after transfer of the hairy roots to PC medium, they dedifferentiated to cells and formed a suspension culture. After five subcultures, the cells were filtered and transferred to solid B5 medium, and the suspension cells regenerated root tissue. A single root from a petri dish was transferred to a flask, and after several transfers a new hairy root line was established. The redifferentiated (R2) line showed a growth similar to that of the original line with a doubling time of 1.84 days, as compared to the 1.74 days of the control and exhibited active growth with extensive lateral branching similar to that of the control. Southern blot analysis showed that the R2 line was also transformed (data not shown).

Transformed roots of *C. roseus* line J1 cultured in vitro have a morphology similar to that of the roots of normal plants: both possess a terminal protective root cap composed of several layers of parenchymatous cells. The apical meristem of the root is adjacent to the root cap, and the junction between the cap region and the meristem is composed of cells in a "closed" structure (Fig. 1 A). The root can be divided into three regions, the meristematic region, the differentiating region and the mature region (Fig. 1 A). When the transformed roots were cultivated in the dedifferentiation medium for 24 days, they lost their structure, the epidermis of the root and the meristematic region disappeared and, at the same time, a meristematic zone appeared near to the central cylinder (Fig. 1 B, C). This meristematic zone grew and formed a lateral root which gave origin to the dedifferentiated cells. Finally, all the root cells segregated to form a cell suspension culture. Regenerated roots have a similar morphology and structure to those of the original root, the only difference being that the regenerated line has only a few layers of cells in the root cap and this is not-well defined (Fig. 1 D).

Pasquali et al. (1992) showed that the addition of auxins to the medium of *C. roseus* suspension cultures inhibits the transcription of TDC and SS genes. They proposed that auxins function as a switch for active cell division or secondary metabolite synthesis, two processes that have repeatedly and quite consistently been reported to be inversely related (Lindsey and Yeoman 1983).

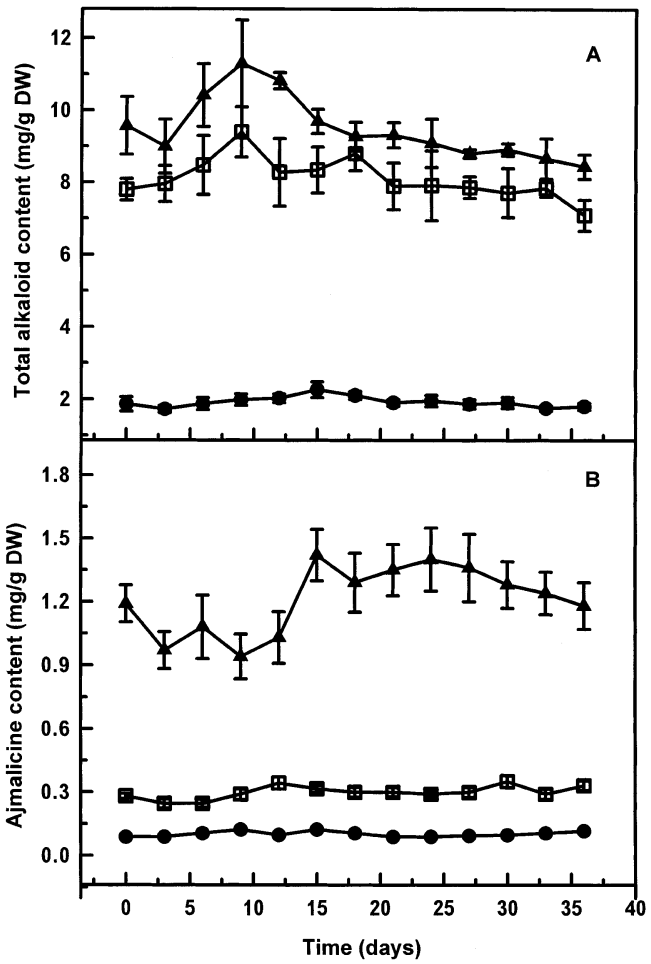

Fig. 2 A, B Effect of level of differentiation on total indole alkaloid and ajmalicine accumulation in *C. roseus*. Three flasks were harvested every 3 days during a culture period of 36 days, and the alkaloids were extracted and quantified as described in the Materials and methods. Data presented are the result of two independent experiments. **A** Total alkaloids, **B** ajmalicine, ▲ Control hairy roots ● cell suspension □ regenerated roots (R2)

Indole alkaloid accumulation

In *C. roseus* seedlings, indole alkaloid biosynthesis is under developmental control (Balsevich et al. 1986; De Luca et al. 1986). When hairy roots were transferred to PC modified medium, the roots gradually lost their capability to synthesise alkaloids. This loss in biosynthetic capability reached 50% (data not show) before the root cultures had developed into a single cell state. The cell suspension generated via dedifferentiation contained a fivefold lower level of alkaloids than the previous hairy root culture (Fig. 2 A). After transfer of the cell suspension onto B5 solid medium, roots were regenerated and, subsequently, the alkaloid content almost returned to that of the control (Fig. 2 A).

Ajmalicine was found to be one the major alkaloids in *C. roseus* hairy roots, line J1 (Ciau-Uitz et al. 1994). However, the ajmalicine content in the cell suspension culture was only 7% of that present in the original root line. When roots had been regenerated, ajmalicine content was only

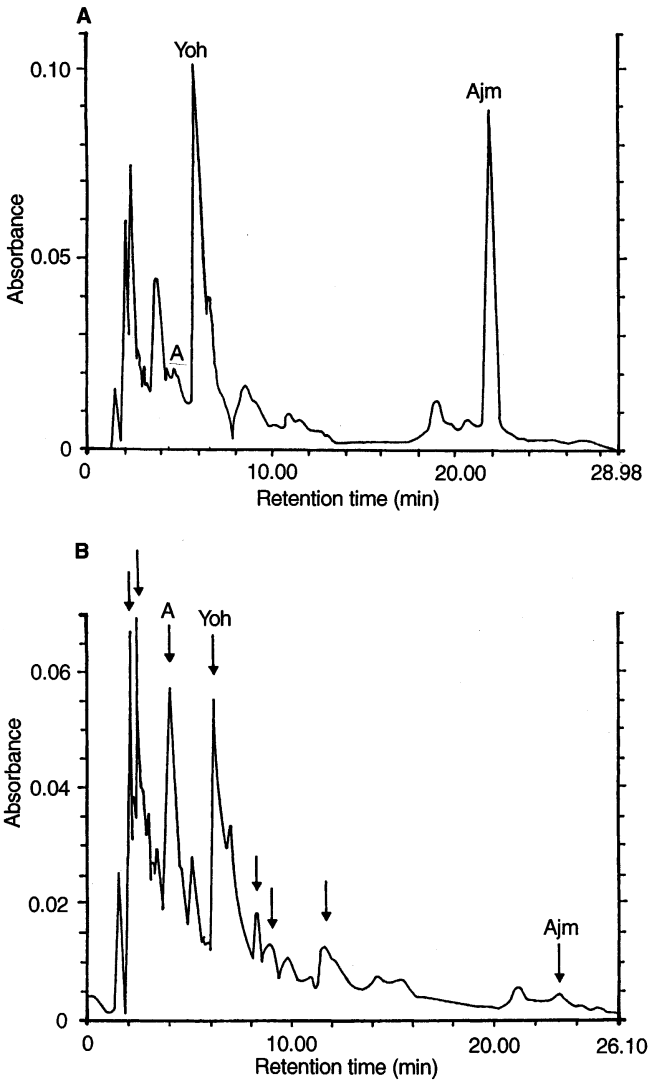

Fig. 3A,B Effect of differentiation on the alkaloid pattern in *C. roseus*. Roots were harvested on day 21 of culture and samples were analysed by HPLC. **A** J1 line, **B** R2 line. *Ajm* ajmalicine, *A* ajmaline; *Yoh* yohimbine. The *arrows* show differences in the patterns of the individual alkaloids between the two lines

20% of that of the control (Fig. 2B). Since alkaloid accumulation had been restored, the next question was if the qualitative pattern had been conserved. We observed that the individual alkaloid pattern changed dramatically during the dedifferentiation-differentiation process. In control hairy roots, ajmalicine and yohimbine were two of the major alkaloids; however, in the regenerated lines the levels of these two alkaloids had decreased considerably, with ajmalicine being present only in trace amounts. On the other hand, ajmaline as well as some unidentified alkaloids had increased in the regenerated line (Fig. 3).

TDC activity

Alkaloids are affected both quantitatively and qualitatively during differentiation-dedifferentiation, possibly as a result of a change in some key enzymes. The activity of TDC, the enzyme that converts tryptophan into tryptamine, is highly regulated by the developmental processes of *C. roseus* seedlings (De Luca et al. 1988). In our experiment TDC activity decreased by at least one order of magnitude when the hairy roots dedifferentiated into suspension cells (Fig. 4). However, during the period that the level of the alkaloids was recovering, when on B5 medium the roots regenerated from cells, TDC activity remained low and reached only 5% of that of the control (Fig. 4).

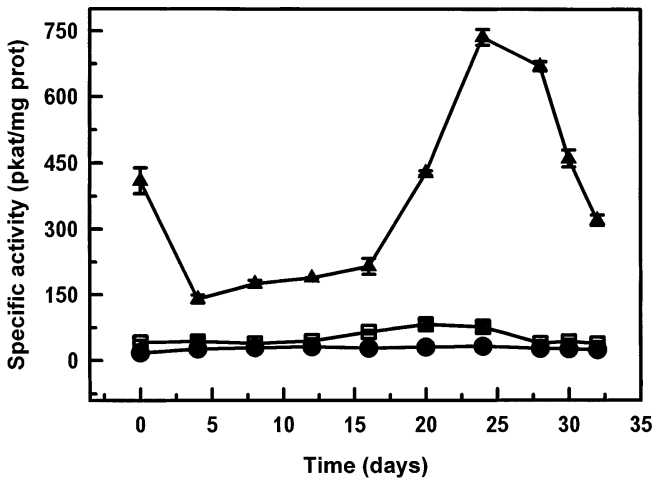

Fig. 4 Effect of differentiation on tryptophan decarboxylase activity in *C. roseus*. Three flasks were harvested every 3 days during the culture period and the enzyme activity was quantified as described in the Materials and methods. Data presented are the results of two independent experiments. ▲ Control hairy roots, ● cell suspension, □ regenerated roots, R2

SS activity

Strictosidine synthase activity decreased when the roots dedifferentiated to cells (Fig. 5) and only recovered to 30% of the initial activity when the cells were regenerated into roots (Fig. 5).

HMGR activity

Evidence suggests that HMGR is one of the key regulatory mechanisms that control carbon flow in indole alkaloid biosynthesis (Stermer and Bostock 1987; Chappell and Nable 1987). Here, HMGR activity was low during the cell suspension stage; however, unlike TDC and SS enzyme activities, HMGR enzyme activity completely recovered to control levels when the roots were regenerated (Fig. 6).

The regulation of some enzyme activities in the indole alkaloid pathway depends on the stage of development of the plants. In *C. roseus* and *Cinchona ledgeriana*, enzyme activity of the TDC and the SS are under strict developmental control (Balsevich et al. 1986; De Luca et al. 1988; Aerts et al. 1990, 1991). Here, indole alkaloid accumulation and TDC and SS activities were strongly related to the

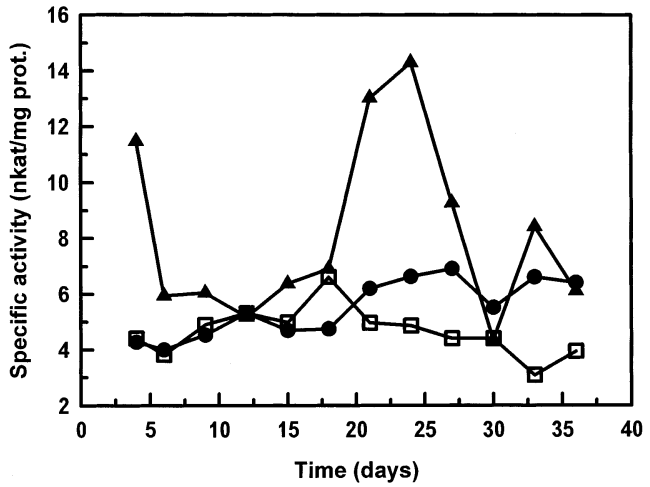

Fig. 5 Effect of differentiation on strictosidine synthase activity in *C. roseus*. Three flasks were harvested every 3 days during the culture period and the enzyme activity was quantified as described in the Materials and methods. Data presented are the results of two independent experiments. ▲ Control hairy roots, ● cell suspension cultures, □ regenerated roots, R2

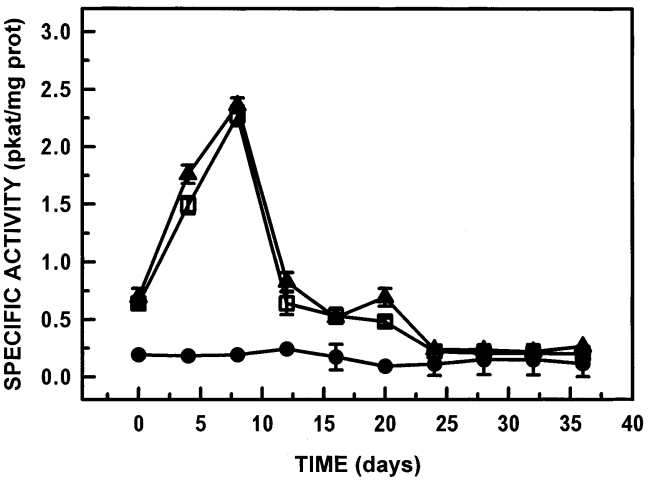

Fig. 6 Effect of differentiation on 3-hydroxy-3-methyl glutaryl Coenzyme A reductase activity in *C. roseus*. Three flasks were harvested every 3 days during the culture period and the enzyme activity was quantified as described in the Materials and methods. Data presented are the results of two independent experiments. ▲ Control hairy roots, ● cell suspension, □ regenerated roots, R2

degree of differentiation. Remarkably, Figs. 4 and 5 show that 5% of control TDC activity was enough to produce a total indole alkaloid level of 8–9 mg/g DW. This result and the results obtained by Knobloch and Berlin (1983) suggest that TDC is not the rate-limiting step in alkaloid biosynthesis and that the regulation may reside in the terpenic pathway

SS enzyme activity showed the same pattern as that of the TDC activity. Roewer et al. (1992) demonstrated a transient and very similar induction pattern between TDC and SS transcripts in *C. roseus* cell culture, after elicitor treatment, and that this pathway is co-ordinately regulated.

Recently we found that the pattern of SS isoenzymes changes over the period of a hairy root culture (Galaz-Avalos and Loyola-Vargas, unpublished data). If the 30% of SS enzyme activity that remains in hairy roots regenerated from cells relative to the level found in initial hairy roots reflects the activity of some isoenzymes rather than that of the total enzymes, then specific individual metabolic channels may be involved. Furthermore, every such channel might lead to the formation of one specific alkaloid. Studies are underway to look into these possibilities.

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