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Transient induction of tryptophan decarboxylase (TDC) and strictosidine synthase (SS) genes in cell suspension cultures of *Catharanthus roseus*

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Summary: When cell suspension cultures of *Catharanthus roseus* are treated with autoclaved elicitor from the fungus *Pythium aphanidermatum*, they respond with the rapid transient induction of tryptophan decarboxylase (TDC) and strictosidine synthase (SS) enzyme activities, followed by the accumulation of indole alkaloids (Eilert *et al.*, 1987). In this report, we demonstrate that expression of TDC and SS enzyme activities is preceeded by the transient appearance of mRNAs for both enzymes, suggesting transcriptional control of these events. The strong transient accumulation of both TDC and SS enzyme transcripts observed in elicitor-treated cell suspension cultures contrasts with the barely detectable level of TDC transcripts and the undetectable level of SS transcripts observed in developing seedlings.

Abbreviations: TDC, tryptophan decarboxylase; SS, strictosidine synthase

Key words: *Catharanthus roseus*-elicitors-tryptophan decarboxylase-strictosidine synthase-transcriptional activation

Introduction:

The enzymes tryptophan decarboxylase (TDC; EC 4.1.1.27) and strictosidine synthase (SS; EC 4.3.3.2), respectively, catalyse the decarboxylation of tryptophan to tryptamine and the stereospecific condensation of tryptamine and secologanin to form the indole alkaloid glucoside strictosidine (Fig. 1). Strictosidine occupies a central role in the biosynthesis of the major classes of monoterpenoid indole alkaloids of *Catharanthus roseus* as well as in the other members of four plant families (Apocynaceae, Loganiaceae, Rubiaceae and Nyssaceae) which produce these compounds (Scott et al. 1981, Stöckigt 1980).

Recent studies with cell suspension cultures of *Catharanthus roseus* have established that indole alkaloid biosynthesis can be transiently induced by changes in nutrient composition (Knobloch et al. 1981), or by treatment of cultures with biotic or abiotic elicitors (Eilert et al. 1987). The stimulating effects of these treatments on indole alkaloid production often

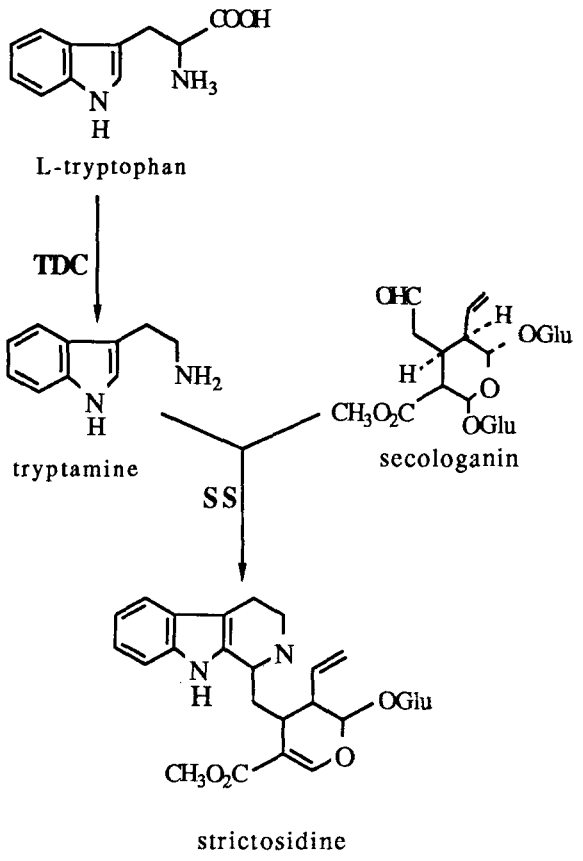

Fig. 1 Enzyme reactions catalysed by tryptophan decarboxylase (TDC) and strictosidine synthase (SS).

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resulted from the induction of pathway-specific enzymes, including TDC and SS. However, the strict correlation between the appearance of TDC and SS enzyme activities and alkaloid accumulation was not consistently found in cell suspension cultures, which suggested that neither enzyme catalysed rate-limiting steps in these artificial systems.

Studies with developing seedlings of *Catharanthus roseus* (Balsevich et al. 1986, De Luca et al. 1986, De Luca et al. 1988) and of *Cinchona ledgeriana* (Aerts et al. 1990, Aerts et al. 1991) showed that TDC and SS enzyme activities as well as the rest of the respective pathways for indole alkaloid and for quinoline alkaloid biosynthesis were under strict developmental and environmental control. SS enzyme activity appeared early after germination and the enzyme was present throughout seedling growth, whereas TDC enzyme activity appeared for a brief 48 h period which coincided with the appearance of alkaloids in *Catharanthus* (De Luca et al. 1986) and in *Cinchona* (Aerts et al. 1990) seedlings. These results suggested that in contrast to the situation in the *Catharanthus* tissue culture system, alkaloid accumulation in intact seedlings was regulated by the appearance of tryptophan decarboxylase enzyme activity.

In this report we use cDNA clones of both TDC (De Luca et al. 1989) and SS (McNight et al. 1990) to characterize the expression of the TDC and SS gene in plant cell suspension cultures and in developing seedlings of *Catharanthus roseus*.

Materials and Methods:

Plant material. Cell suspension cultures of Catharanthus roseus (cell line # 615) were grown in 100 ml of B5 medium (according Eilert et al. 1987) in dim light at 26±1 ° C with agitation at 150 rpm. Fungal elicitor (Pythium aphanidermatum) homogenate was prepared as reported previously (Eilert et al. 1987). Autoclaved Pythium homogenate (5 % v/v) was added to an appropriate number of cultures at day 2, 4 and 6 as specified under Results. Cultures were harvested by filtration 0, 4, 8, 12, 24 and 48 h after elicitor application. Non-elicited control cultures were harvested at day 1, 2, 3, 4, 5, 6, 8, 13 and 33 after transfer of stationary phase cultures to fresh B5 medium. Harvested cells were weighed, frozen in liquid nitrogen and stored at -80 ° C.

Growth of seedlings. Seedlings of Catharanthus roseus (L.) G. Don cv Little Delicata were surface sterilized and were grown in the dark as described previously (De Luca et al. 1986). Between 20 to 35 g of seedlings per day were harvested, frozen in liquid nitrogen and stored at -80 ° C.

RNA isolation. Total RNAs were extracted from plant tissues as described previously (De Luca et al. 1989). Tissues were homogenized using a mortar and pestle in the presence of liquid nitrogen until a fine powder was obtained. Extraction buffer (2.25 ml/g tissue) containing 100 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 % SDS and phenol/ chloroform/isoamyl alcohol 25:24:21 (v/v/v) (1.5 ml/g tissue) was added to the powder; the resulting slurry was transferred to sterile centrifuge tubes and shaken vigorously for 15 min. The aqueous phase was separated from the organic phase by centrifugation for 10 min at 10,000 rpm at 4 ° C. Total RNA was extracted by standard procedures (Jones et al. 1985) and Poly(A)$^+$ mRNA was isolated by chromatography on oligo(dT)-cellulose (Aviv and Leder 1972).

Northern hybridizations. RNA samples were submitted to electrophoresis on 1 % agarose gels containing 2.2 M formaldehyde according to Maniatis et al (1982). The gels were washed with sterile-distilled water, followed by a solution of 50 mM NaOH and 10mM NaCl and neutralization with a solution of 100 mM Tris-HCl pH 7.5. The transfer of RNA to Gene Screen membranes (New England Nuclear Research Products) was carried out in 20xSSC using a vacuum blotting pump (Pharmacia PL Biochemicals, Baie d'Urfée, Québec). The membranes were incubated for 5 h at 42 ° C (Bellco hybridizaion oven) in prehybridization buffer (5x Denhardt's, 50 % deionized formamide, 5xSSPE, 0.1 % SDS, 0.1 mg/ml herring sperm DNA).

RNA blot hybridization was performed with a [^{32}P]-labelled 1600 bp EcoR1 fragment from TDC (accession # J04521) cDNA insert or with a [^{32}P]-labelled 1000 bp BamH1 fragment from SS (accession # X53602) cDNA insert. Autoradiograms were optically scanned using a Hoeffer GS 300 scanning densitometer.

Results:

Accumulation of TDC transcripts in cell suspension cultures and in developing seedlings.

In order to study the kinetics of TDC transcript appearance, cells were harvested throughout the growth cycle of the *Catharanthus* cell line # 615. Very low levels of TDC transcripts could be detected throughout growth of this cell line on 1B5 medium (Fig 2a, Days 1 to 33).

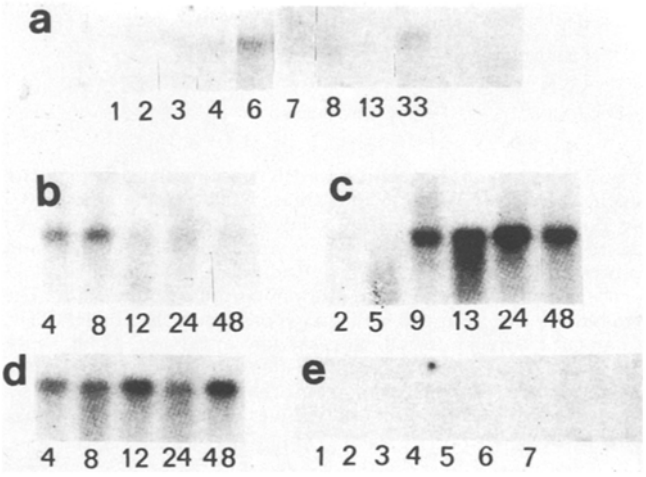

Fig. 2 Kinetics of TDC mRNA accumulation, a) during growth of cell line # 615 after transfer of cells to 1B5 medium, b)-d) during elicitor treatment or e) during C. roseus seedling growth. a) TDC mRNA accumulation in 1, 2, 3, 4, 6, 8, 13 & 33 day old cell suspension cultures. b) 2 day old cells were treated with elicitor and TDC mRNA accumulation is measured at 4, 8, 12, 24 & 48 h after treatment. c) 4-day old cells were treated with elicitor and TDC mRNA accumulation was measured at 2, 5, 9, 13, 24 & 48 h after treatment. d) 6-day old cells were treated with elicitor and TDC mRNA accumulation is measured at 4, 8, 12, 24 & 48 h after treatment. e) TDC mRNA accumulation is measured at days 1, 2, 3, 4, 5, 6 & 7 of seedling development. Samples of 3.3 µg of Poly (A)$^+$RNA were applied on 1% Agarose gels and were processed as described in Materials and Methods. After hybridization with the labelled TDC probe, the membrane was autoradiographed for 24 h.

Treatment of cells with *Pythium* elicitor after 2, 4 or 6 days of growth resulted in rapid transient induction of TDC transcripts irrespective of cell age (Fig 2b-d). The most intense induction of TDC transcripts occurred in 4-day old *Pythium*-treated cells.

The appearance of TDC transcripts could not be detected during seedling development (Fig 2e). Overexposure of this blot for 6 days, however, resulted in the detection by densitometry of a 6-fold increase in TDC transcripts by days 3 and 4 over day 2 of seedling development and which decreased by 50 % by day 6 of seedling development (data not shown).

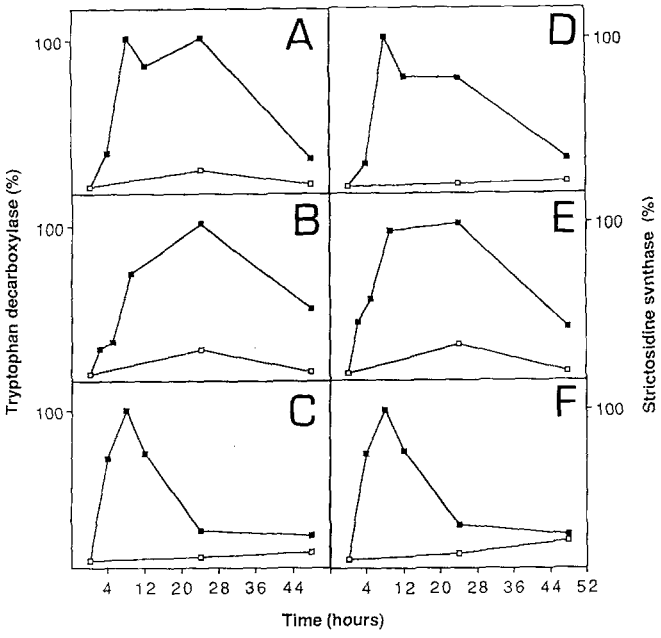

Fig. 3 Kinetics of TDC and SS mRNA accumulation in control unelicited cells (□) and elicitor treated cells (■).Samples of 3.3 µg Poly (A)$^+$RNA from 2 (A & D), 4 (B & E) and 6 (C & F) day old cells plus treatments were applied on 1% agarose and processed as in Fig. 2. After hybrididization with labelled SS probe, the membrane was autoradiographed for 17 h. The membrane was stripped and was reprobed with labelled TDC fragment. After autoradiography for a further 17 h, both autoradiograms were scanned by densitometry and the data point which gave the maximum absorbance in each panel was set at a value of 100 % and all other values were set as a percentage of the data point.

Accumulation of SS transcripts in cell suspension cultures and in developing seedlings:

The previous results raised a question concerning the appearance of other biosynthetic pathway enzymes. The availability of cDNA clones for SS permitted RNA blot hybridization to measure the appearance of SS transcripts in cell suspension cultures and in developing seedlings. No SS transcripts could be detected during growth of cells on 1B5 medium (Fig 3 D-F) nor during seedling development (data not shown). As discovered for TDC, the appearance of SS transcripts could be induced after treatment of 2, 4, or 6 day old cell suspension cultures with *Pythium* elicitor (Fig 3 D-F). The patterns of appearance of elicitor-induced transcripts for TDC (Fig 3 A-C) and for SS (Fig 3 D-F) were very similar between elicitor treatments of 2, 4 or 6 day old cultures. The increases of TDC and SS transcripts were first detected 4 h after elicitor treatment and the relative levels of elicitor-induced transcripts increased with the age of the cultures (Fig 3 C, F). The mRNA levels for TDC and SS rose to a maximum in the first 8 to 24 h after elicitor treatment of 2 and 4 day old cell suspension cultures (Fig 3 A, B, D, E), whereas they rose rapidly to a maximum by 8 h and had fallen by 24 h of elicitor treatment of 6 day old cell suspension cultures.

Discussion:

Plant cell suspension cultures have the ability to respond to external stimuli with changes in gene expression that result in altered secondary metabolism. Previous studies (Eilert et al. 1987) have demonstrated that elicitor treatment of *Catharanthus roseus* cell suspension cultures resulted in transient induction of TDC and SS enzyme activities followed by accumulation of indole alkaloids. Pretreatment of cell suspension cultures with cycloheximide, an inhibitor of translation, prevented the *Pythium*-induced increase of TDC and SS specific activities as well as indole alkaloid accumulation. The studies of Noé and Berlin (1985) showed that the induction of TDC enzyme activity occurring after transfer of cells to an alkaloid production medium was due to an increase in the TDC transcripts which they measured by immunoprecipitation of TDC *in vitro* translation products.

In this report we demonstrate that the elicitor-mediated induction of TDC and SS enzyme activities followed by the accumulation of indole alkaloids occur as a result of specific increases of TDC and SS mRNAs. When cells growing on 1B5 medium are treated with fungal elicitors, they respond by the transient production of mRNA transcripts for TDC and SS (Fig 2 & 3). The elicitor-mediated induction of TDC and SS transcripts is observed after treatment of 2, 4 and 6 day old cell suspension cultures. The mRNAs for both TDC and SS reaches a maximum 8-24 h after elicitor application (Fig 3) which slightly preceeds the induction of TDC and SS enzyme activities whose maxima occurr 12-36 h after elicitor treatment (Eilert et al 1987)). The time course of appearance of TDC and SS transcripts is also in agreement with the complete release from inhibition of elicitor-induced alkaloid

accumulation which is observed when cycloheximide is added 12 h after elicitor treatment (Eilert et al. 1987). The transient and very similar induction pattern observed for both TDC and SS transcripts (Fig 3) in this *Catharanthus* cell culture suggests that this pathway is coordinately regulated and that elicitor-mediated TDC and SS gene activation is controlled by some common mechanism.

Our previous studies gave ambiguous results when transcriptional and translational inhibitors strongly decreased elicitor-mediated TDC enzyme induction, but had less effect on SS enzyme activity (Eilert et al 1987). The recent isolation of SS cDNA clones from *Rauvolfia serpentina* (Kutchan et al 1989) and from *Catharanthus roseus* (McNight et al. 1990) identified the presence of a signal peptide which correctly targeted this protein to the plant vacuole in a tobacco heterologous system (McNight et al 1991). The vacuolar localisation of SS could, therefore, contribute to the different patterns of SS enzyme activities observed with inhibitors.

Surprisingly, poly(A)$^+$RNA isolated from developing seedlings revealed no hybridization with TDC (Fig 2) and SS (data not shown) cDNA clones. The appearance of TDC but not SS mRNA could be detected only after prolonged exposure of mRNA blots. The appearance of TDC mRNA in developing seedlings by day 3 of seedling development and it's 50% decrease by day 6 of seedling development coincides well with the previously reported time course for immunological detection of TDC protein followed by the appearance of enzyme activity (Fernandez et al 1989) and the developmentally regulated expression of these two enzyme activities.

The specific mRNAs encoding TDC and SS enzyme activities appear to be present in very low abundance in developing seedlings as compared with the high levels of transcripts which appear in elicitor-induced cell suspension cultures. The expression of TDC and SS enzyme activities in both systems, however, appears to be induced in a similar manner. In addition, the development-specific appearance of TDC enzyme activity in seedlings of *Catharanthus roseus* (Fernandez et al 1989) and in *Cinchona ledgeriana* (Aerts et al 1990) was always accompanied by the production and accumulation of alkaloids, whereas cell suspension cultures selected for increased TDC enzyme activity did not necessarily produce indole alkaloids (Knobloch et al 1981). The ability to coordinately induce the accumulation of high levels of TDC and SS transcripts by elicitor treatment of cell suspension cultures will be used to isolate regulatory factors which control expression of these two genes.

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