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Coordinated regulation of two indole alkaloid biosynthetic genes from *Catharanthus roseus* by auxin and elicitors

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Abstract

Catharanthus roseus (periwinkle) produces a wide range of terpenoid indole alkaloids, including several pharmaceutically important compounds, from the intermediate strictosidine. The complete mRNA sequence for the enzyme strictosidine synthase (SSS) was determined. Comparison of the primary structure of the encoded protein with the amino-terminal sequence of purified SSS indicated the presence of a signal peptide of 31 amino acids in the putative primary translation product. SSS is encoded by a single-copy gene indicating that isoenzymes reported by others are formed post-translationally from a single precursor. The *sss* gene and the tryptophan decarboxylase gene (*tdc*), encoding another enzyme essential for indole alkaloid biosynthesis, are coordinately regulated. In plants steady-state mRNA levels are highest in roots. In cell suspension cultures the genes are rapidly down-regulated by auxin. In contrast, both genes are strongly induced by fungal elicitors such as *Pythium aphanidermatum* culture filtrate or yeast extract. Induction is a rapid, transcriptional event occurring independent of *de novo* protein synthesis. These results show that a first important regulatory step in the complex process leading to indole alkaloid accumulation in *C. roseus* suspension cells is transcription of the biosynthetic genes.

Introduction

Plants produce a wide variety of secondary metabolites that are not absolutely essential for the survival of individual cells but are thought to contribute to the overall fitness of the organism. The biosynthesis of these compounds is often restricted to certain cell types and may in addition be induced by environmental stimuli, such as pathogenic infection, pathogenesis-related signal

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X61932.

molecules termed elicitors, certain chemicals or UV light. For the phenylpropanoid pathway this induction was shown to be at least partly due to enhanced transcription of the genes involved [6, 12, 14, 15, 17, 29].

Catharanthus roseus or Madagascar periwinkle belongs to the family Apocynaceae, which produces a class of secondary metabolites termed terpenoid indole alkaloids. Possible functions of these compounds include antimicrobial or antifeeding activity, UV protection or nitrogen storage and transport. Their synthesis has mainly been studied out of pharmaceutical interest. The root-derived monomeric alkaloids ajmalicine and serpentine are used in the treatment of cardiac and circulatory diseases, and the leaf-derived dimeric alkaloids vinblastine and vincristine are potent anti-tumour drugs. Terpenoid indole alkaloids are formed via branches of two primary metabolic pathways, the shikimate and the mevalonate pathways. Tryptophan decarboxylase (TDC, EC 4.1.1.28) catalyses the conversion of L-tryptophan into tryptamine, and geraniol-10-hydroxylase (G10H) is involved in the conversion of the essential oil geraniol into the monoterpenoid glucoside secologanin. Strictosidine synthase (SSS, EC 4.3.3.2.) catalyses the condensation of tryptamine and secologanin to form strictosidine, the universal precursor for terpenoid indole alkaloids in plants (Fig. 1).

TDC and SSS have been purified from *C. roseus* [21, 26] and partial cDNA sequences have been reported [4, 19]. Little is known about the regulation of *tdc* and *sss* gene expression. *tdc* mRNA is induced by transfer of suspension cells to a so-called production medium, one of the characteristics of which is the absence of phytohormones [20]. Auxin was found to downregulate *tdc* transcription in cultured cells [9]. Synthesis of SSS enzyme is also induced by transfer of suspension cells to a hormone-free production medium [7], but it is unknown whether auxin is involved. *De novo* synthesis of TDC and SSS enzymes in suspension cells is stimulated by fungal elicitors [7]. Whether elicitor induction operates at the transcriptional level or post-transcriptionally is unknown.

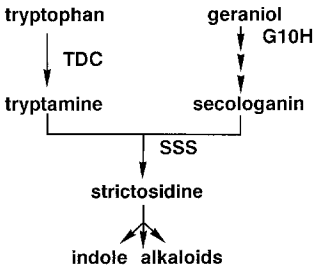

Fig. 1. Biosynthesis of terpenoid indole alkaloids. The enzymes indicated are: TDC, tryptophan decarboxylase; G10H, geraniol-10-hydroxylase; SSS, strictosidine synthase.

Here we report the complete mRNA sequence for SSS from *C. roseus*. We show that it is encoded by a single gene, which is expressed coordinately with the single-copy *tdc* gene in plants and cell suspensions. The transcriptional regulation of both genes by auxin and elicitors is described.

Material and methods

Plant material

Catharanthus roseus (L.) G. Don. plants (cv. Morning Mist, seed from Kieft, Blokker, Netherlands) were grown in a greenhouse at 23 °C. Cell suspension cultures of *C. roseus* line MP183L were grown in 57.5 ml of LS medium [16] containing 2 mg/l 1-naphthaleneacetic acid (NAA) and 0.2 mg/l kinetin in 250 ml Erlenmeyer flasks in continuous light (TLF65W33, 1000–1200 lx; Philips) at 25 °C on a gyratory shaker with an orbit of 2.54 cm at 150 rpm. Subculturing was done every 7 days by 8-fold dilution in 50 ml of fresh medium. Cells were collected by vacuum filtration on an 80 μm nylon filter, frozen in liquid nitrogen and stored at −80 °C.

Elicitor preparation and induction

Pythium elicitor was prepared by autoclaving a filtered (0.2 μm) *Pythium aphanidermatum* culture grown in 100 ml of LS medium for 6 days at 27 °C

after inoculation with 1 cm^2 of fresh mycelium. Yeast extract (Difco) was dissolved in water and autoclaved. Cellulase from *Aspergillus niger* (Fluka) and salicylic acid (Janssen) were dissolved in water, the pH was adjusted to 5.8 with KOH and the solutions were filter-sterilized. Crab shell chitosan (Sigma) was dissolved in 10% acetic acid, the pH was adjusted to 5.8 with NaOH and the solution was filter-sterilized. *C. roseus* cultures were cultured for 10–12 days in LS medium with hormones, subsequently diluted 8-fold in the same medium, and 10 ml of *Pythium* culture filtrate or 0.5 ml of other elicitors were added between 0 and 24 h before harvesting at day 3. This procedure gave low background levels of *sss* and *tdc* mRNAs. Cycloheximide was added 10 min prior to yeast extract to a final concentration of 25 μM.

Anti-SSS antiserum preparation and N-terminal sequencing of SSS

SSS was purified as described (A. de Waal *et al.*, manuscript in preparation) and 70 μg were emulsified with 600 μl of Freund's complete adjuvant (Gibco) and injected subcutaneously in a New Zealand white rabbit. Booster injections containing 35 μg of SSS emulsified with 600 μl of Freund's incomplete adjuvant were administered 3 times at 2-week intervals. Blood collected 2 weeks after the last injection was left at room temperature for several hours and centrifuged at 2000 rpm for 30 min. The clear supernatant was stored at −20 °C. Testing of anti-SSS antiserum by ELISA gave a titer of 10^{-4}. For protein sequencing 512 pmol of purified SSS was electrophoresed by SDS-PAGE with thioglycolic acid in the upper buffer compartment to prevent N-terminal blocking and transferred to an Immobilon PVDF (Millipore) membrane. The SSS band was visualized by amido-black staining, excised and N-terminally sequenced by Volker Kruft at the Max-Planck-Institute for Molecular Genetics, Berlin, Germany.

Extraction and western blotting of plant proteins

Proteins were extracted from plant tissue powdered in liquid nitrogen by boiling for 5 min in 50 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol. Insoluble material was pelleted in a microfuge. Protein concentrations were estimated by Coomassie brilliant blue (CBB) staining of gel-electrophoresed samples, since the high SDS and 2-mercaptoethanol levels did not allow a Bradford protein determination. For western blot analysis identical amounts of CBB-stainable protein (about 1 μg) were separated by SDS-PAGE and transferred to Immobilon PVDF. The membrane was stained after incubation with anti-SSS antiserum as described for cDNA library screening.

cDNA library construction and screening

A cDNA library consisting of 1.7×10^6 primary recombinants was constructed according to the ZAP-cDNA Synthesis protocol (Stratagene) using 5 μg of poly(A) RNA isolated from roots of 3-month-old *C. roseus* plants and amplified. Nitrocellulose filters (Schleicher & Schuell BA85) containing 400000 plaques were screened with 1000-fold diluted anti-SSS antiserum preincubated with 0.5 mg/ml *Escherichia coli* strain XL-1 protein extract according to the Protoblot Immunoscreening System Manual (Promega). Using the isolated cDNA as a probe nylon filters (Hybond-N, Amersham) containing 750000 plaques were screened in an attempt to isolate a full-length clone. Isolated cDNA fragment was ^{32}P-labelled by random hexamer primer extension with Klenow DNA polymerase. (Pre)hybridization and washing of the filters was done as described for Southern blots.

Plant nucleic acid isolation

Total RNA was isolated from tissue ground in liquid nitrogen by phenol/chloroform extraction and precipitation with LiCl at a final concentra-

tion of 2 M. Plant DNA was purified from the supernatant by isopropanol precipitation and CsCl-EtBr centrifugation. Poly(A) RNA was isolated using Poly(A) Quick columns (Stratagene).

Nucleic acid sequencing

Sequencing of (subcloned) cDNA inserts in pBluescript SK (Stratagene) or M13 tg130/131 was done with sequenase (USB). The longest clone pCCR38 was sequenced on both strands. The 5′ end of *sss* mRNA was directly sequenced as described [8] using 10 μg poly(A) RNA and the oligonucleotide 5′-CTGCCATCATGG-3′.

Nucleic acid sequence data were analysed with the University of Wisconsin Genetics Computer Group Sequence Analysis Software Package [5].

Southern and northern blot analysis

Digested DNAs were electrophoresed in 1% agarose/TBE gels and transferred to GeneScreen (NEN) membranes by capillary blotting with 0.5 M NaOH. Total RNA (10 μg/lane) was electrophoresed in 1.5% agarose/formaldehyde gels as described [1] and transferred to GeneScreen membranes by capillary blotting with 50 mM sodium phosphate, 5 mM EDTA pH 6.5. Baked blots were (pre)-hybridized in 50% deionized formamide, 5× SSPE, 5% SDS at 42 °C, washed with 0.1× SSPE, 0.5% SDS at 65 °C and exposed to Fuji-RX films mounted on Kyokko intensifying screens at −80 °C.

Run-off transcription

Nuclei were isolated from fresh *C. roseus* cells essentially as described [30]. The yield was 0.5–1×10^5 nuclei/g fresh weight of cells. Run-off transcription was performed in 250 μl containing $1–2 \times 10^6$ nuclei and 250 μCi [^{32}P]α-UTP [30]. Labelled RNA extracted according to [10] was resuspended in hybridization buffer and 0.5–1×10^7 cpm were hybridized to Southern blots containing 2 μg of cDNA clones digested with restriction enzymes to excise the insert.

Results

Determination of the complete mRNA sequence for SSS

Strictosidine synthase (SSS) was purified from a *C. roseus* cell suspension culture, which is a good source of SSS enzyme activity [25]. Western blotting with rabbit anti-SSS antiserum showed that it recognized a protein of expected size in extracts from roots as well as from leaves and cultured cells (Fig. 2).

An amplified cDNA expression library from *C. roseus* roots was screened with this antiserum. Sequencing of a positive cDNA clone indicated that it was partial with a length of 764 bp. The cDNA library was rescreened using this cDNA as a probe. Among 20 positive clones only one was possibly full-length. The sequence of this cDNA clone, called pCCR38, was determined. Direct sequencing of *sss* mRNA by primer extension on poly(A) RNA from elicitor-treated suspension cells showed that pCCR38 missed 72 bp at its 5′ end (result not shown). The 5′-terminal nucleotides could not be determined unambiguously due to bands in all lanes representing mRNA 5′ end-points. The identity of these nucleotides was confirmed by comparison with the

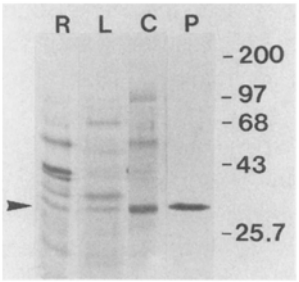

Fig. 2. SSS is detectable in different *C. roseus* tissues. A western blot with protein extracted from roots (R), leaves (L) or suspension-cultured cells (C) was probed with anti-SSS antiserum. Lane P contains 50 ng of purified SSS running at the position indicated with an arrowhead. Sizes of marker proteins ($\times 10^{-3}$) are indicated.

corresponding genomic sequence (G. Pasquali and J. Memelink, unpublished results).

The complete sequence of *sss* mRNA deduced from pCCR38 and direct mRNA sequencing is shown in Fig. 3. It has a length of 1280 nt excluding the poly(A) tail and contains an open reading frame (ORF) encoding a protein of 352 amino acids (aa) with M_r 39093.

The first AUG codon fits the plant consensus sequence for translation start sites [3]. Comparison of the protein deduced from the mRNA sequence with the N-terminal sequence of the purified SSS protein used to raise antibodies (Fig. 3) showed that the putative primary translation product is processed by removal of the N-terminal 31 aa. The cleavage site was correctly predicted by McKnight *et al.* [19]. The size of the processed protein deduced from the sequence corresponds closely to the molecular weight of 36000 calculated from western blots for purified SSS (Fig. 2). Several differences were observed in comparison with a previously reported *sss* cDNA sequence [19] as recorded in the EMBL Database. Most importantly the reported sequence misses the 5′ end including the first 9 codons and it has a frameshift in the 3′ end of the ORF leading to a C-terminal extension of 5 aa compared to our sequence. Also 2 nucleotide differences are present within the ORF which lead to aa alterations. Our sequence contains 19 additional nucleotides immediately upstream of the poly(A) tail. Another cDNA clone sequenced terminates at the same position (No. 1262 in Fig. 3) as the previously reported sequence, indicating that the *sss* mRNA population is heterogeneous regarding the 3′ end.

Estimation of sss *gene copy number*

To estimate the copy number of the *sss* gene in the *C. roseus* genome, Southern blot analysis was carried out (Fig. 4). Total genomic DNA from *C. roseus* digested with restriction endonucleases was hybridized with the pCCR38 cDNA insert. One band was observed with *Hind* III, and two bands with *Eco* RI or *Bgl* II, which is consistent with the notion that in the haploid genome *sss* is

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   1  TATCAAACCATACCCCATAGCTGCCCCTCTCTGCTTCACTCCCATCATTT
  51  ACAGTCAACAATGGCAAACTTTTCTGAATCTAAATCCATGATGGCAGTTT
            M  A  N  F  S  E  S  K  S  M  M  A  V  F
 101  TCTTCATGTTTTTCCTTCTTCTTCTTTCTTCTTCTTCTTCTTCTTCTTCT
       F  M  F  F  L  L  L  L  S  S  S  S  S  S  S  S
 151  TCTTCACCAATTTTGAAAAAGATTTTTATTGAAAGCCCTTCCTATGCTCC
       S  S  P  I  L  K  K  I  F  I  E  S  P  S  Y  A  P
 201  GAATGCCTTCACCTTCGATTCAACTGATAAAGGGTTCTACACTTCCGTCC
       N  A  F  T  F  D  S  T  D  K  G  F  Y  T  S  V  Q
 251  AAGATGGCCGAGTTATCAAATATGAAGGGCCAAATTCAGGCTTCACTGAC
       D  G  R  V  I  K  Y  E  G  P  N  S  G  F  T  D
 301  TTCGCCTACGCATCTCCCTTCTGGAACAAAGCTTTTTGTGAGAACAGCAC
       F  A  Y  A  S  P  F  W  N  K  A  F  C  E  N  S  T
 351  CGATCCAGAGAAAAGACCATTGTGTGGGAGGACATATGATATTTCCTATG
       D  P  E  K  R  P  L  C  G  R  T  Y  D  I  S  Y  D
 401  ACTATAAGAACAGCCAAATGTACATTGTTGATGGCCATTACCATCTTTGT
       Y  K  N  S  Q  M  Y  I  V  D  G  H  Y  H  L  C
 451  GTGGTTGGAAAAGAAGGTGGGTATGCCACACAACTAGCCACAAGTGTGCA
       V  V  G  K  E  G  G  Y  A  T  Q  L  A  T  S  V  Q
 501  AGGAGTGCCATTCAAATGGCTCTATGCAGTAACTGTTGATCAGAGAACAG
       G  V  P  F  K  W  L  Y  A  V  T  V  D  Q  R  T  G
 551  GGATTGTTTATTTCACTGATGTTAGCTCCATACATGATGACAGTCCCGAA
       I  V  Y  F  T  D  V  S  S  I  H  D  D  S  P  E
 601  GGTGTGGAAGAAATCATGAATACAAGTGATAGAACAGGGAGATTAATGAA
       G  V  E  E  I  M  N  T  S  D  R  T  G  R  L  M  K
 651  GTATGATCCTTCAACAAAAGAAACCACCTTATTATTGAAAGAGCTACATG
       Y  D  P  S  T  K  E  T  T  L  L  L  K  E  L  H  V
 701  TTCCCGGCGGTGCAGAAATCAGCGCAGATGGTTCCTTTGTTGTAGTAGCA
       P  G  G  A  E  I  S  A  D  G  S  F  V  V  V  A
 751  GAATTTTTAAGCAATCGGATAGTGAAGTATTGGCTAGAAGGGCCAAAGAA
       E  F  L  S  N  R  I  V  K  Y  W  L  E  G  P  K  K
 801  AGGCAGTGCAGAGTTCTTAGTTACAATCCCAAATCCAGGAAATATAAAGA
       G  S  A  E  F  L  V  T  I  P  N  P  G  N  I  K  R
 851  GGAATTCTGATGGCCATTTTTGGGTGTCTTCAAGTGAAGAATTAGATGGA
       N  S  D  G  H  F  W  V  S  S  S  E  E  L  D  G
 901  GGTCAACATGGAAGAGTTGTTTCAAGAGGAATTAAGTTTGATGGATTTGG
       G  Q  H  G  R  V  V  S  R  G  I  K  F  D  G  F  G
 951  GAATATTCTTCAAGTTATACCACTTCCACCACCATATGAAGGTGAACATT
       N  I  L  Q  V  I  P  L  P  P  P  Y  E  G  E  H  F
1001  TTGAACAGATTCAAGAGCACGATGGTTTGTTATACATTGGAAGTCTCTTC
       E  Q  I  Q  E  H  D  G  L  L  Y  I  G  S  L  F
1051  CATAGCTCTGTGGGTATATTAGTGTATGATGATCATGATAACAAGGGAAA
       H  S  S  V  G  I  L  V  Y  D  D  H  D  N  K  G  N
1101  TTCTTATGTTTCTAGCTAGTCATTAATTGAATATTTGTTTTGATTTGTAA
       S  Y  V  S  S  *
1151  CTTTTTAATGATTTCTGCAAATTCTATGGCTTTTTGAAGGTTACAAAGTC
1201  TTAGGAGGAGATTTTGAGTGTCTGCTGCTACATATATGTTCAATAAGAAG
1251  CCAATTCCATGAATTACGTGTTAGGGTTTT
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Fig. 3. Nucleotide sequence of *sss* mRNA and amino acid sequence of the encoded protein. The amino acids determined by N-terminal sequencing of purified SSS are underlined. The signal peptide cleavage site is indicated with an arrowhead. The sequence complementary to the primer used for direct mRNA sequencing is overlined. A putative polyadenylation signal is underlined. cDNA clone pCCR38 starts at the nucleotide indicated with an arrow.

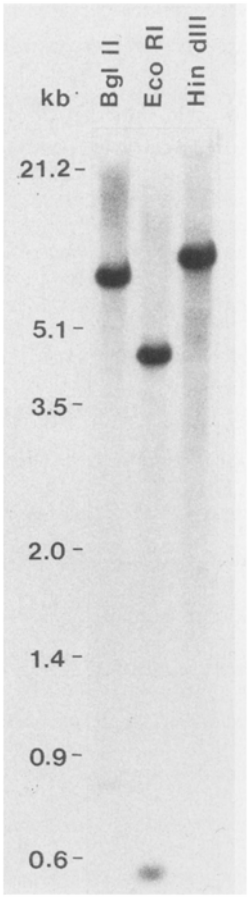

Fig. 4. Southern blot of *C. roseus* DNA probed with *sss* cDNA. Ten μg of DNA was digested with *Bgl* II, *Eco* RI or *Hin*d III. Sizes of marker fragments are indicated in kb.

a single-copy gene containing one internal *Eco* RI site in an exon (position 853 in Fig. 3) and one internal *Bgl* II site in an intron (G. Pasquali and J. Memelink, unpublished results). The observation that the partial sequences determined from 11 independent cDNA clones are identical (results not shown) and the finding that 7 independent overlapping genomic *sss* clones have identical restriction maps (G. Pasquali and J. Memelink, unpublished results) corroborate this assumption.

sss *and* tdc *expression in plants*

The expression of *sss* in plants was compared with the expression of the *tdc* gene. A cDNA clone (pCCR41) of *tdc* which was almost identical to the previously reported *tdc* clone [4] was isolated from the root cDNA library. Total RNA from roots, stems, leaves and flowers of 3-month-old plants was extracted and northern blot analysis was carried out. The result (Fig. 5) shows that, for both *sss* and *tdc* genes, steady-state mRNA levels are highest in roots, with much less expression in leaves. Low but detectable levels were found in stems and flowers. For *sss* the mRNA differences between leaves and roots are not reflected in the protein amount (cf. Figs. 2 and 5). One possible explanation for this apparent discrepancy is that the SSS protein is relatively stable.

sss *and* tdc *expression in cultured cells: effect of auxin*

Suspension cells were routinely cultured in LS medium containing both cytokinin (0.2 mg/l kinetin) and auxin (2 mg/l NAA). The *tdc* gene was demonstrated to be transcriptionally down-regulated by auxin, while cytokinin had no apparent effect [9]. As found previously for *tdc* [9], subculturing in hormone-free medium for 3 days also induced *sss* expression (Fig. 6A). As a control the blots were probed with cDNA clone pCCR12, hybridizing to an abundant mRNA of around 700 nucleotides of unknown identity. The level of

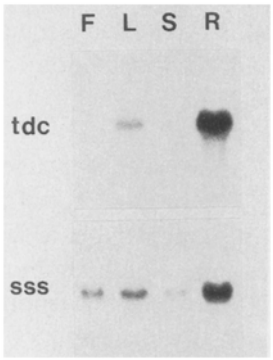

Fig. 5. Expression of *sss* and *tdc* in plants. Gel blots containing total RNA from flowers (F), leaves (L), stems (S) and roots (R) of 3-month-old plants were probed with *sss* and *tdc* cDNAs.

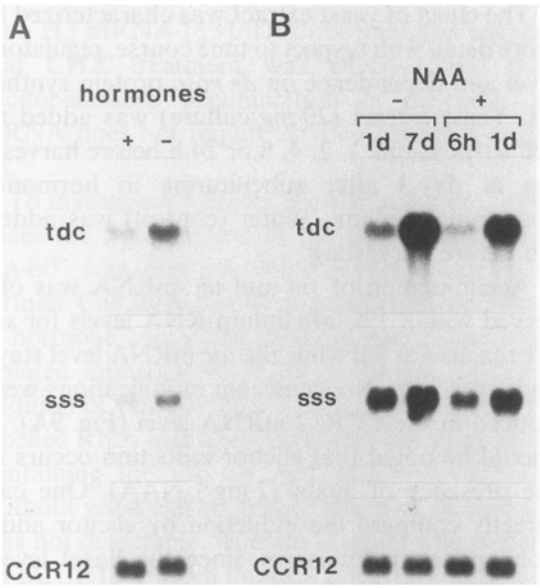

Fig. 6. Negative regulation of *sss* and *tdc* mRNA levels by auxin. A. Suspension cells were cultured for 3 days in the presence (+) or absence (−) of hormones. B. Suspension cells were cultured from 1 (1d) up to 7 (7d) days in the absence (−) of hormones, and after 7 days of hormone starvation NAA was added (+) for 6 hours (6h) up to 1 day (1d). Gel blots containing total RNA were probed with *sss*, *tdc* and CCR12 cDNAs.

CCR12 mRNA was not affected by omission of hormones.

Next, the effect of auxin on *sss* expression was determined and compared with the regulation of *tdc*. Suspension cells were transferred to hormone-free medium and NAA (final concentration of 10 μM) was added after 7 days. Total RNA was extracted 1 and 7 days after subculturing in hormone-free medium and 6 and 24 h after NAA addition. As shown in Fig. 6B, both *sss* and *tdc* genes are induced after transfer to the hormone-free medium with high levels of steady-state mRNA at day 7. After addition of NAA these mRNA levels decrease within 6 h. For *tdc* the effect is much more dramatic than for *sss*, which could be due to differences in mRNA stability. The decrease is transient and 24 h after NAA addition the original high levels are almost completely restored. This rapid mRNA accumulation following the initial drop could be due to NAA uptake and metabolism by the hormone-starved cells. Thus auxin has a negative regulatory effect on both *sss* and *tdc* gene expression.

Run-off transcription experiments were carried out to investigate whether this down-regulation is transcriptional or entirely due to changes in mRNA stability. Suspension cells were grown for 7 days in hormone-free medium. Nuclei were isolated 3 h after addition of NAA to a final concentration of 10 μM or water (control) to the cultures. The ^{32}P-labelled RNA made during *in vitro* run-off transcription was hybridized with Southern blots containing the cDNAs for *tdc*, *sss* and the constitutively expressed mRNA CCR12. Figure 7 shows clear signals for all cDNAs with RNA from the hormone-starved nuclei. After addition of NAA transcription of *sss* and *tdc* is undetectable. NAA has no effect on the transcription of CCR12 gene(s).

These experiments demonstrate that both the *sss* and *tdc* genes are transcriptionally down-regulated by auxin.

sss *and* tdc *expression in cultured cells: effect of elicitors*

Terpenoid indole alkaloids and corresponding enzyme activities in *C. roseus* were shown to be induced by fungal elicitors [7]. Therefore, diverse elicitors were screened to evaluate their effect on the expression of the *sss* and *tdc* genes. Cell suspensions were subcultured in LS medium con-

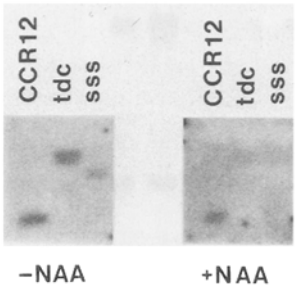

Fig. 7. Transcriptional downregulation of *sss* and *tdc* genes by auxin. Radiolabelled run-off transcripts synthesized by nuclei isolated from hormone-starved cultures that were treated with water (−NAA) or exposed to NAA for 3 h (+NAA) were hybridized to gel blots containing *sss*, *tdc* and CCR12 cDNAs.

taining both cytokinin (0.2 mg/l kinetin) and auxin (2 mg/l NAA). Cellulase (10 mg/50 ml culture), *Pythium aphanidermatum* elicitor (10 ml autoclaved culture filtrate/culture), yeast extract (20 mg/culture), salicylic acid (0.01, 0.1 or 1 mM final concentration) or chitosan (0.2, 1.5 or 8 mg/culture) were added 1, 8 or 24 h before harvesting at day 3 after subculturing.

Chitosan and salicylic acid were found to have weak inducing effects on *sss* and *tdc* steady-state mRNA levels 8–24 h after exposure of the cells to 1.5 mg/culture and 0.1 mM, respectively. Cellulase was found to mediate a relatively strong induction of both mRNAs (results not shown). However the most effective inducers at the exposure times and concentrations tested proved to be *Pythium* culture filtrate and yeast extract (Fig. 8), with some induction within 1 h and maximally induced levels of *sss* and *tdc* mRNA at 8 h after addition. The steady-state mRNA levels of both genes decreased 24 h after the addition of *Pythium* culture filtrate but they stayed relatively high when yeast extract was added.

The effect of yeast extract was characterized in more detail with respect to time course, regulatory level and dependence on *de novo* protein synthesis. Yeast extract (20 mg/culture) was added to cell suspensions 1, 2, 4, 8 or 24 h before harvesting at day 3 after subculturing in hormone-containing medium. Water (control) was added 4 h before harvesting.

Accumulation of *sss* and *tdc* mRNA was observed within 1 h. Maximum RNA levels for *sss* are reached at 8 h while the *tdc* mRNA level stays high until 24 h. No significant modifications were noticed in the CCR12 mRNA level (Fig. 9A). It should be noted that elicitor induction occurs in the presence of auxin (2 mg/l NAA). One can directly compare the induction by elicitor addition and auxin omission since the basal levels shown for auxin (Fig. 6A, + lane) and elicitor (Figs. 8 and 9, W lane) were measured in identically treated cultures.

Dependence on *de novo* protein synthesis was tested by cycloheximide (CHX) addition 10 min before the addition of yeast extract. Treatments

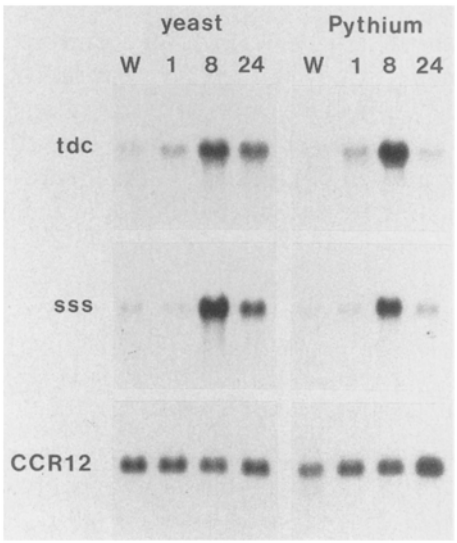

Fig. 8. Positive regulation of *sss* and *tdc* mRNA levels by elicitors. Suspension cells were exposed for 1, 8 or 24 h to yeast extract or *Pythium aphanidermatum* culture filtrate. Control cultures were incubated for 24 h with water (W). Gel blots containing total RNA were probed with *sss*, *tdc* and CCR12 cDNAs.

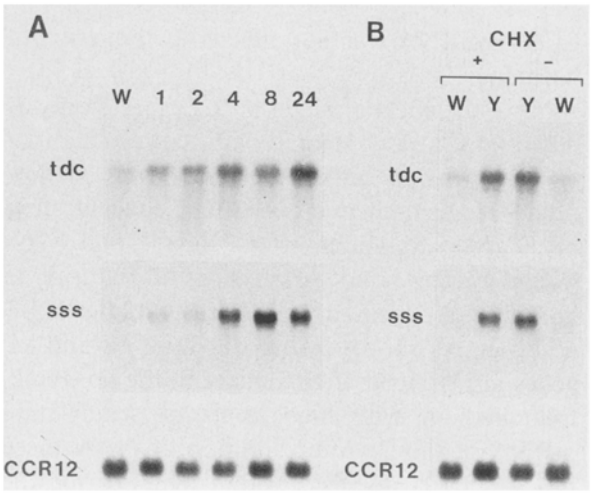

Fig. 9. Time course (panel A) and cycloheximide independence (panel B) of *sss* and *tdc* mRNA induction by yeast extract. A. Suspension cells were exposed for 1, 2, 4, 8 or 24 h to yeast extract. A control culture was incubated for 4 h with water (W). B. Suspension cells were exposed for 4 h to water (W) or yeast extract (Y), alone (–) or in combination (+) with cycloheximide (CHX). Gel blots containing total RNA were probed with *sss*, *tdc* or CCR12 cDNAs.

were done for 4 h before harvesting at day 3 of subculturing. CHX had no influence on the steady-state mRNA levels of *sss*, *tdc* or CCR12 in control or elicitor-treated cultures (Fig. 9B). As a control for the effectivity of the CHX treatment northern blot analysis with the same RNA samples using a soybean histone H4 cDNA clone as a probe was carried out. CHX caused a significant increase in the steady-state level of the histone H4 mRNA (results not shown). This result is in agreement with studies on HeLa cells showing that inhibition of protein synthesis induces histone H3 and H4 transcription and mRNA stabilization [28], and studies with wheat seedlings showing that CHX causes accumulation of histone H3 mRNA [13]. Our observations suggest that CHX treatment was effective and that elicitor-induced accumulation of *tdc* and *sss* mRNA does not require *de novo* protein synthesis.

To investigate whether elicitor treatment influences the transcription of the *tdc* and *sss* genes run-off transcription experiments were performed. Cell suspensions were cultured for 5 days in hormone-containing medium and nuclei were isolated immediately or 15 or 30 min after the addition of yeast extract. *In vitro* transcribed ^{32}P-labelled RNA was hybridized with Southern blots containing the *sss*, *tdc* and pCCR12 cDNAs. Without yeast extract transcription of *sss* and *tdc* was not detectable, whereas a signal was evident for pCCR12. Fifteen minutes after the addition of yeast extract a clear signal was already observed for *sss* and *tdc* and the transcriptional induction of both genes is even more evident after 30 min (Fig. 10). These results indicate that the induction of the *sss* and *tdc* genes by yeast extract is a very rapid effect (within 15 min) occurring at the transcriptional level in a process where no *de novo* protein synthesis is required.

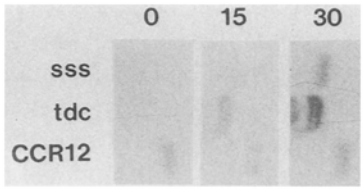

Fig. 10. Transcriptional induction of *sss* and *tdc* genes by elicitor. Radiolabelled run-off transcripts synthesized by nuclei isolated from suspension cultures that were untreated (0) or exposed for 15 or 30 min to yeast extract were hybridized to gel blots containing *sss*, *tdc* and CCR12 cDNAs.

Discussion

We have determined the complete mRNA sequence coding for the enzyme strictosidine synthase from *Catharanthus roseus* and have studied the regulation of *sss* expression in relation to the expression of *tdc*, another gene essential for indole alkaloid biosynthesis.

The data presented here indicate that SSS is encoded by a single-copy gene and therefore the reported SSS isoenzymes [26] are probably generated post-translationally from a single precursor. The predicted presence of a signal peptide [19] was confirmed by the N-terminal amino acid sequencing of purified SSS. SSS is located in the vacuole [18] and the signal peptide most likely is necessary for correct targeting. The vacuolar location of SSS from *C. roseus* after overexpression in tobacco depends on the integrity of the signal peptide [18]. The occurrence of SSS in the vacuole suggests that this organelle is a site of alkaloid biosynthesis and not merely a storage compartment. In this context it is interesting that the interconversion of the alkaloids ajmalicine and serpentine was recently shown to occur in the vacuole [2].

Elicitors rapidly induce the transcription of the *tdc* and *sss* genes without a need for *de novo* protein synthesis. In contrast, auxin down-regulates the transcription of both genes. Additional experiments with *tdc* have shown that this down-regulation is very rapid (occurring within 15 min) and independent of *de novo* protein synthesis [9]. Auxin is an essential growth regulator in the medium necessary to overcome an unknown rate-limiting step in cell division. Thus auxin appears to function as a switch between active cell division or secondary metabolite formation, two processes that have repeatedly and quite consistently

been reported to be inversely related [11, reviewed in 27].

Since *tdc* (O.J.M. Goddijn *et al.*, unpublished results) and *sss* are single-copy genes, the different regulatory pathways must operate on single promoter regions. The transcriptional responses to elicitors and auxin are rapid and independent of *de novo* protein synthesis suggesting that these signal molecules modulate the activity of pre-existing transcription factors, for instance by (de)-phosphorylation. An interesting question is whether the pathway for auxin-down-regulation uses the same signal transduction components, for instance the same receptor, as the pathway for auxin-induced gene expression [reviewed in 24]. Another question raised by the finding that elicitors have inducing activity in the presence of auxin is how elicitor-mediated signal transduction processes operate in relation to auxin-activated events.

The regulation of indole alkaloid biosynthetic genes in *C. roseus* strongly resembles the well-studied regulation of several genes in phenylpropanoid metabolism in a variety of plant species. The phenylalanine ammonia-lyase, 4-coumarate: CoA ligase, chalcone synthase and chalcone isomerase genes are rapidly induced by elicitors at the transcriptional level [6, 15, 17, 29]. In addition, PAL [22, 23] and CHS [22] mRNA levels were shown to be negatively regulated by auxin in carrot suspension cells.

One could envisage that plants switch from primary to secondary metabolism by upregulating key genes operating at the boundary between these two pathways (such as *tdc* or PAL), while further downstream genes (such as *sss* or 4-CL) are more or less constitutively active. Alternatively, all of the specific genes in the secondary pathway could be upregulated in a coordinated fashion. The latter model seems to apply for both phenylpropanoid and indole alkaloid biosynthesis. Since the genes are coordinately regulated it is likely that their promoters bind a similar set of transcription factors. Hence plants switch to secondary metabolism by modulating the activities of the transcription factors involved.

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