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CELL BIOLOGY AND MORPHOGENESIS

# Transcript profiling of terpenoid indole alkaloid pathway genes and regulators reveals strong expression of repressors in *Catharanthus roseus* cell cultures

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**Abstract** The understanding of the complexities and molecular events regulating genes and the activators involved in terpenoid indole alkaloid (TIA) metabolism is known to a certain extent in cell cultures of an important TIA yielding plant, *Catharanthus roseus*, though it is not yet complete. Recently, the repressors of early TIA pathway genes have also been identified. However, their roles in the regulation of TIA pathway in *C. roseus* cell cultures remains yet unknown. We have made a comparative profiling of genes catalyzing the important steps of 2-C methyl-D-erythritol-4-phosphate (MEP), shikimate and TIA biosynthetic pathways, their activator and repressors using macroarray, semiquantitative RT-PCR and northern analyses in a rotation culture system of *C. roseus* comprising differentiated and proliferated cells. Our results demonstrate that TIA biosynthetic pathway genes and their activators show variable expression pattern, which was correlated with the changes in the cellular conditions in these systems. Under similar conditions, TIA pathway repressors show strong and consistent expression. The role of repressors in the complex regulation of the TIA pathway in *C. roseus* cell cultures is discussed. The results were supported by HPLC data, which demonstrated that the molecular program of cellular differentiation is intimately linked with TIA pathway gene expression and TIA production in *C. roseus* cell cultures.

**Keywords** *Catharanthus roseus* · Primary and TIA pathway transcripts · Rotation cell cultures · TIA pathway repressors · TIAs

**Abbreviations**

| | |
|---|---|
| BAP | Benzylamino purine |
| CCC | Compact callus cluster |
| 2, 4-D | 2, 4-Dichlorophenoxy acetic acid |
| HPLC | High performance liquid chromatography |
| MeJA | Methyl ester of jasmonic acid |
| MEP | 2-C-methyl-D-erythritol-4-phosphate |
| ORCA | Octadecanoid-derivative responsive *Catharanthus* AP2-domain protein |
| R-CCC | Reverted-compact callus cluster |
| RT-PCR | Reverse transcriptase-polymerase chain reaction |
| S | Suspension |
| TIA | Terpenoid indole alkaloid |
| YE | Yeast extract |

## Introduction

Terpenoid indole alkaloids (TIAs), an important group of secondary metabolites are produced by *Catharanthus roseus* (L.) G. Don (Madagascar periwinkle), a member of the apocynaceae family. Some of these

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alkaloids have high therapeutic values such as, the antihypertensive alkaloids, ajmalicine, serpentine and anticancer alkaloids, vincristine, vinblastine (van der Heijden et al. 2004). The monoterpenoid moiety of TIAs originates from the 2-C methyl erythritol phosphate (MEP) pathway whereas the indole skeleton of TIAs is derived from the shikimate pathway (Fig. 1). The biosynthesis of TIAs in *C. roseus* is reported to be under strict regulation. Their metabolism has been found to be restricted to certain tissues and it is modulated by developmental and environmental mechanisms (van der Fits and Memelink 2000; Endt et al. 2002). Despite significant efforts, TIA biosynthesis in plant cell cultures remains poorly characterized (De Luca and Laflamme 2001). Regulation of two important TIA biosynthetic pathway genes, namely, strictosidine synthase (*Str*) and tryptophan decarboxylase (*Tdc*) has been studied in great detail, which revealed thereof coordinate regulation and elicitor-mediated induction in cell cultures (Pasquali et al. 1992; Menke et al. 1999a, b; van der Fits and Memelink 2000; Siberil et al. 2001). The regulatory mechanisms controlling many of the remaining steps of the TIA pathway are yet unknown, the understanding of which may reveal the complex molecular program that governs TIA metabolism in cell culture. Functional analysis of *Str* and *Tdc* gene promoters led to the identification of *cis*-regulatory sequences involved in elicitor and MeJA-induced responses (Menke et al. 1999b; Ouwerkerk et al. 1999; Pasquali et al. 1999). Octadecanoid-derivative responsive *Catharanthus* AP2-domain (ORCA3) transcription factor was found to be jasmonate-responsive transcriptional regulator of *C. roseus* primary and secondary metabolism (van der Fits and Memelink 2000). Furthermore, G-box binding factors (GBF1, GBF2 and GBF3) (Siberil et al. 2001) and zinc finger proteins (ZBF1, ZBF2 and ZBF3) (Pauw et al. 2004) were shown to act as repressors in the regulation of elicitor-induced TIA metabolism in *C. roseus*. Two downstream vindoline pathway genes, desacetoxyvindoline 4-hydroxylase (*D4h*) and deacetoxyvindoline 4-O-acetyltransferase (*Dat*) are known to be transcriptionally blocked in *C. roseus* in vitro grown cultures (Vazquez-Flota et al. 2002). Many aspects of such key

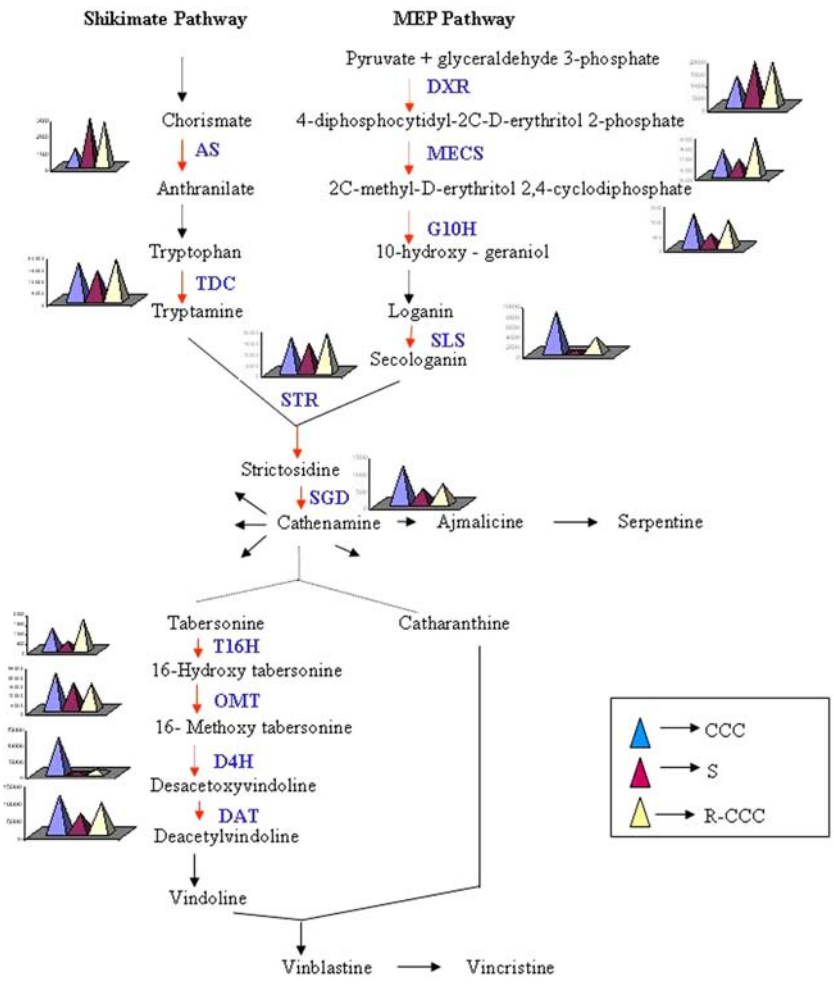


**Fig. 1** TIA biosynthesis in *C. roseus*. *Solid arrows* indicate single step whereas *broken arrows* represent multiple-step enzymatic conversions. Genes investigated in this study are shown by *bold arrows*. Graphical representations of fold-level expression of respective gene as obtained by semiquantitative RT-PCR analysis of leaf-induced CCC, S and R-CCC cultures are shown beside each enzymatic step

regulatory events have been recently explored (van der Heijden et al. 2004; Rischer et al. 2006), but understanding of the regulation of complete TIA pathway in cell cultures is still lacking for which it has not yet become a viable alternative for improved production of TIAs.

The differential expression of genes in different state of cultures is expected to be different and will also be associated with different levels of secondary metabolites. In order to demonstrate the status of similar changes in expression of TIA pathway genes and regulators in *C. roseus* cell culture, we have devised a rotation culture system and studied the comparative transcript profiles of TIA and related primary pathway genes, activator and repressors in this system. The rotation culture system consisted of a leaf and root-induced compact callus cluster (CCC) culture followed by initiation of cell suspension (S) and then transferring the proliferated cells once again back to compact callus cluster culture (R-CCC) condition. This system provided alternating conditions of cellular differentiation and proliferation and thus allowed to study the transcriptional changes of TIA pathway genes and regulators simultaneously under changing culture conditions. The results were compared with TIA production data by HPLC. This study demonstrates the complexities of molecular regulation of TIA metabolism in *C. roseus* cell culture and indicates the probable bottleneck for scale-up production of TIAs in this system.

## Material and methods

### Cell cultures

Compact callus cluster (CCC) culture was grown from leaf and root tissues of 6–8 weeks old *C. roseus* var. Prabal seedlings (Dutta et al. 2005) on Gamborg's basal medium (Gamborg et al. 1968) supplemented with 1 PPM 2, 4-dichlorophenoxyacetic acid (2, 4-D) and 0.1 PPM benzylaminopurine (BAP) (BD3) (Sigma Chemicals, USA). Cultures were maintained by transferring approximately 1 g (fresh weight) of compact callus in 50 ml of fresh solid medium regularly at every 21 days interval, which constituted a single passage. Suspension (S) culture was initiated by transferring 1 g CCC culture (fresh weight) from the third passage of its growth to 50 ml liquid Gamborg's basal medium supplemented with 0.5 PPM 2,4-D and 0.1 PPM BAP (BD11) in 250 ml Erlenmeyer flask and kept on a gyratory shaker (120 rpm). S cultures were maintained by transferring approximately 10 ml suspension in 50 ml of fresh liquid medium regularly at every 7 days interval. S cultures at 8th–10th passages of growth were allowed to settle down at the bottom of the flask and the liquid medium was decanted off. Settled cells/very small aggregates were transferred back to solid BD3 media and termed as reverted compact callus cluster (R-CCC) culture. R-CCC culture was maintained similarly as CCC culture. The pH of the media was adjusted to 5.8 before autoclaving. For CCC and R-CCC cultures, the media were solidified with 0.3% phytagel (Sigma Chemicals, USA). Cultures were maintained at 25 ± 1°C under 3,800 μmol/$m^2$/$s^1$ light (Philips cool white fluorescent light) for 16/8 h light and dark photoperiod.

### Histological analysis of in vitro grown cells

Tissue samples from 3 months old leaf-induced CCC, R-CCC cultures and 7 days old S culture were fixed overnight in ''acetic acid : absolute ethanol (1:3, v/v)'' at room temperature and stained with 1% aceto-orcein solution for histological analysis by microscopy.

### Construction of cDNA macroarray

We selected 12 genes catalyzing specifically important steps of TIA and related primary pathways, namely, shikimate and MEP, and eight regulators of TIA pathway to construct a macroarray specific for the study of gene expression responsible for TIA production. In addition, we used actin cDNA from *C. roseus* (*CrActin*) as positive and neomycin phosphotransferase II (*npt*II) from vector pBI121 as negative controls as indicated in Table S1. The selected genes were amplified using gene specific primers and checked on 1% agarose gel to confirm the transcript size, quality and quantity. Purified PCR products were denatured by adding 0.6 N NaOH. Approximately 50 ng of PCR products were spotted onto ''8.5–11.5 cm'' Hybond N membrane (Amersham Pharmacia Biotech, Uppsala) using BIO-DOT Blotter (BIO-RAD, USA). The membranes were neutralized with neutralization buffer (0.5 M Tris-HCl, pH 7.4, 1.5 M NaCl) for 3 min, washed with 2 × SSC and crosslinked using UV cross linker (Stratagene, La Jolla, CA, USA).

### Total RNA and mRNA extraction

Tissue samples from leaf-derived CCC, S and R-CCC cultures were ground to powder with liquid nitrogen. Total RNA was extracted from 1 g of tissue (fresh weight) by LiCl method (Dutta et al. 2005). mRNA was isolated from 250 μg total RNA using mRNA isolation kit (Roche, Mannheim, Germany) according to the

manufacturer's instructions. Total RNA and mRNA were quantified using GeneQuant Pro Spectrophotometer (Amersham Pharmacia Biotech, Uppsala, Sweden) and checked on 1.5% denaturing agarose gel.

### Probe preparation and hybridization

The mRNAs from leaf-derived CCC, S and R-CCC cultures were labeled with [α- $^{32}$P] dCTP by first strand reverse transcription. Five-hundred nanograms of mRNA was labeled in a 20 µl reaction volume using SuperScript III reverse transcriptase (Life technologies, Grand Islands, NY, USA). Radiolabelled cDNAs were purified by Sephadex G-50 columns (Sigma, USA). Hybridizations were carried out for 16 h at 55°C in buffer containing 0.5 M sodium phosphate (pH 7.2), 7% (w/v) SDS and 1 mM EDTA (pH 8.0). Membranes were washed in 2 × SSC and 0.1 % (w/v) SDS at room temperature for 10 min followed by washing the filters in 0.5 × SSC and 0.1% (w/v) SDS wash buffer at 55°C for 15 min followed by autoradiographic detection of the hybridized signals. Images of the membranes were scanned using Fluro-S Multimager (BIO-RAD, USA).

### Semiquantitative reverse transcriptase: PCR (RT-PCR) and image analyses

For semiquantitative RT-PCR analysis, total RNA was isolated from leaf, root, CCC, S and R-CCC tissues. For reverse transcription, first strand cDNA was synthesized from 5 µg DNase treated total RNA using oligo $dT_{(15)}$ primer (Promega, Madison, USA) and SuperScript III reverse transcriptase. The second strand cDNA was amplified using gene specific primer from 2 µl of first strand cDNA as template in 25 µl reaction volume. For TIA pathway gene clones, the open reading frames were amplified by PCR using either full length or internal primer pairs (Sigma Genosys, USA). Amplification involved 30 cycles of PCR reaction and the cycling conditions were: 30 s denaturation at 94°C, 50 s annealing at 65°C followed by 1 min extension at 72°C for ten cycles, 30 s denaturation at 94°C, 50 s annealing at 60°C followed by 1 min extension at 72°C for ten cycles, 30 s denaturation at 94°C, 50 s annealing at 58°C followed by 1 min extension at 72°C for ten cycles. Optimal cycle number in the linear range for each target gene was determined empirically (Marone et al. 2001). PCR amplification of commonly used house keeping gene actin from *C. roseus* (*CrActin*) was used as a loading control (Fukao et al. 2006) and experiment was carried out in triplicate. The PCR product (25 µl) was loaded on 1% agarose gel and compared. RT-PCR images were obtained with AlphaImager™ 2200 and band intensities were quantified with AlphaImager software (Alpha Innotech Corporation, San Lecandro, CA, USA). Quantitative analysis of semiquantitative RT-PCR images were repeated three times with the cultures generated from leaves of three sets of independently grown *C. roseus* seedlings and the data revealed high degree of reproducibility. To normalize the band intensities of each tissue type, appropriate background value was subtracted. The average of these values from replicated experiments was calculated and resultant value was defined for each target gene.

### Northern analysis

For northern analysis, 20 µg of total RNA from leaf and different leaf-induced cultures were analyzed on 1.5% denaturing agarose gel and transferred to Hybond N membranes following the method reported earlier (Dutta et al. 2005). RNA marker was loaded to confirm the size of the transcripts. Northern blots were hybridized with [α -$^{32}$P] labelled cDNA probes for *Str, Tdc, G10h, Dat, Orca3, Zct1* and *Gbf1*. Hybridizations for *Str* was carried out for 16 h at 65°C in buffer containing 0.5 M sodium phosphate (pH 7.2), 7% (w/v) SDS and 1 mM EDTA (pH 8.0). Membranes were washed in 2 × SSC and 0.1% (w/v) SDS at room temperature for 5 min followed by washing the filters in 0.2 × SSC and 0.1% (w/v) SDS twice at 42°C for 15 min. Hybridizations for rest of the transcripts were performed at 60°C for 16 h in the same hybridization buffer as mentioned before. Membranes were washed initially in 2 × SSC and 0.1% (w/v) SDS at 60°C for 15 min and then with 0.2 × SSC and 0.1% (w/v) SDS at 60°C. The membranes were allowed to dry at room temperature prior to the autoradiographic detection of the hybridized signals. Radioactive images were scanned with a high-resolution scanner (Fluro-S Multimager, BIO-RAD, USA) and northern analysis was performed in triplicate.

### Data acquisition and statistical analysis

Quantitative data for semiquantitative RT-PCR analysis was obtained as described earlier in semiquantitative RT-PCR and image analyses section. The expression profile of primary, TIA pathway genes and regulators were clustered by hierarchical cluster analysis method using cluster program SYSTAT 11 (Eisen et al. 1998). The Spearman correlation and complete linkage methods were used for hierarchical clustering.

### High performance liquid chromatography (HPLC) analysis of TIAs

Total alkaloid was extracted from the leaf-induced CCC, S and R-CCC cultures following the earlier report (Dutta et al. 2005). The tissue samples were harvested at the same time for expression and alkaloid analyses to reduce variation. Chromatographic separation of major indole alkaloids of *C. roseus* was carried out on a reversed-phase C18 column with binary gradient mobile phase profile. The identification of the compounds was based on the retention time and comparison of the UV spectra with those of the authentic standards. Solutions of different authentic samples (catharanthine, vindoline, vinblastine, vincristine, ajmalicine and serpentine) were prepared in methanol (0.25 mg $ml^{-1}$) and used in different concentrations for the preparation of calibration graphs, linear in the range of 0.25–25 μg. Quantification analysis was repeated during the same time as mentioned above for each culture with three replicates and the mean as well as standard deviations were calculated for each experimental sets.

## Results

### Changes in the cellular morphology in CCC, S and R-CCC cultures

CCC culture was initiated from *C. roseus* leaf and root tissues, which consisted mostly of parenchymatous cells ranging from 10 to 20 nm in dimension. In addition, there were xylem and phloem tissues in varying degree in discrete patches (Fig. 2a–c). Thus, CCC culture was heterogeneous in cellular composition. On the other hand, the S culture was homogenous in constitution and consisted of parenchymatous cells only, which were mostly either single round/elongated cell or 2–4 celled globular aggregates (Fig. 2d, e). No single xylem or phloem cell was visible under this condition. Reconverting S to R-CCC culture revealed the cohesion of several parenchymatous cells as well as scattered vascular tissue differentiations (Fig. 2f–h).

### Development of macroarray for profiling of TIA and related primary pathway genes and regulators in cell cultures

The expressions of two feeder primary (MEP, shikimate) and committed TIA metabolite pathway genes were analyzed in *C. roseus* rotation culture system along with TIA pathway regulators using a cDNA array system based on the membrane-supported macroarray technique. The database search for *C. roseus* genome revealed six genes encoding selected enzymes for each of the primary (MEP and shikimate) and TIA pathways along with eight TIA pathway regulator genes. A single amplified product obtained for each target gene was cloned and identities of putative cDNA clones were confirmed by sequencing which showed 95–99% homology with the available sequences in database. The cDNAs were spotted on membrane for macroarray analysis along with *CrActin*

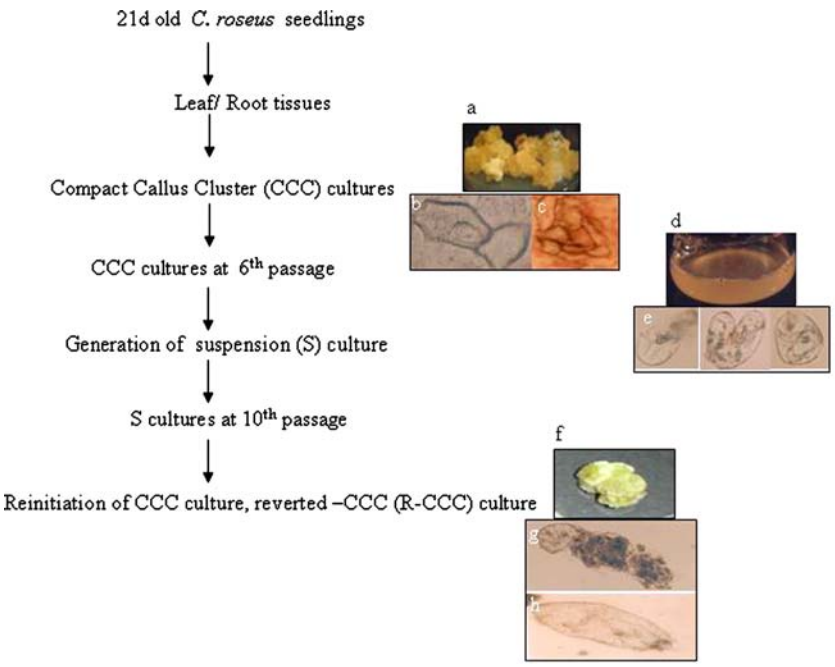


**Fig. 2** Schematic representation of methodology used for development of rotation culture system in *C. roseus* (*left panel*) and comparative histology of the three types of cultures. **a** CCC culture and **b**, **c** xylem differentiation in CCC, **d** S culture and **e** its cell patterns and **f** R-CCC culture and **g**, **h** re-initiation of cellular cohesion and xylem cell in R-CCC cultures

and *NptII* genes as controls (Table S1). Consequently, 22 genes were spotted in duplicate to facilitate the expression analysis and their expressions were monitored in the tissues originating from CCC, S and R-CCC cultures. Each membrane was hybridized simultaneously with cDNA probes synthesized from each sample. There were significant changes in the expression levels of primary as well as TIA pathway genes and the regulators in the samples originating from CCC, S and R-CCC cultures (Fig. S1a–d). TIA pathway repressor genes were, however, relatively highly expressed in the same tissues. This system revealed a high degree of reproducibility.

## Gene, activator and repressor transcripts of TIA and related primary pathways in cell culture system

We compared the transcript profiles of three TIA pathway repressors each of G-box binding factor type, namely, *Gbf1*, *Gbf2* and *Gbf3*, and zinc finger protein type, namely, *Zct1*, *Zct2* and *Zct3*, one master regulator, *Orca3*, one MYB transcription factor, *CrBpf1* and TIA and related primary pathway genes in leaf- as well as root-induced CCC, S and R-CCC cultured cells initially by semiquantitative RT-PCR analysis. The appropriate number of cycles for PCR amplification of each target gene was determined so that it can be quantified and that the amplification lies in the exponential range before reaching the plateau. PCR amplification of 30 cycles was found to be optimum for all the target genes studied and their amplification was found to be in the exponential range (data not shown). The mRNA levels of primary pathway genes, *Mecs* and *Dxr* were higher in leaf-induced CCC, S and R-CCC cultured cells compared to that of the *As*, *G10h* and *Sls* (Fig. 3a). The transcript levels of the latter two genes, *G10h* and *Sls* remained undetected in leaf-induced S cultured cells. The mRNA levels of early TIA pathway genes, *Tdc* and *Str*, showed changes with changing conditions of growth and thus were higher in CCC and R-CCC but low in S conditions. *Sgd* transcript showed low or undetected level of expression under similar conditions. The levels of expressions of late TIA pathway transcripts, *T16h*, *D4h*, *Dat* changed from high to low with changing culture conditions (CCC to S). Some of these show restoration of expression in R-CCC cultures. Similar study was carried out using root-induced CCC, S and R-CCC cultures, which revealed similar pattern of expression for root-specific TIA as well as primary pathway genes except *G10h*, which was faintly detected in this system (Fig. 3b). One gene from each pathway, namely *G10h* (MEP pathway), *Tdc* (shikimate pathway), *Str* (early TIA pathway) and *Dat* (late TIA pathway) were used as probes for northern analysis, which revealed biphasic nature of expression of *Tdc* and *Str* transcripts in leaf-induced CCC, S and R-CCC cultures noted by a reduction in the level of expression from CCC to S, but again the level of expression was restored in R-CCC culture (Fig. 3c). The transcript level of *G10h* was confirmed to be low in S and R-CCC cultured cells and *Dat* transcript remained undetected by northern analysis.

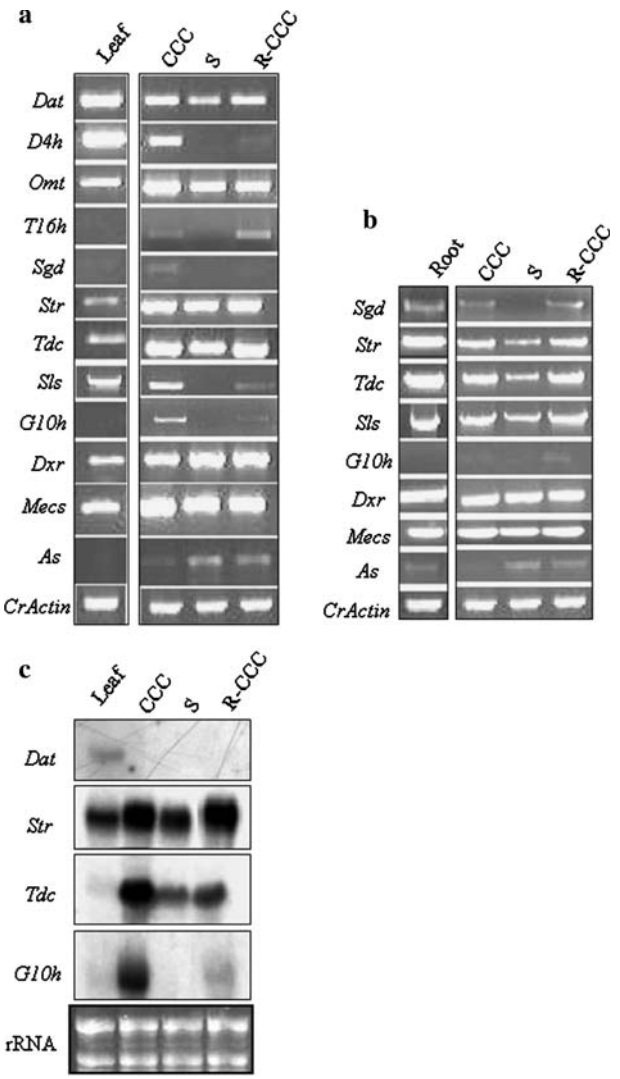


**Fig. 3** Expression patterns of TIA pathway genes by semiquantitative RT-PCR and northern analyses. Semiquantitative RT-PCR analysis of **a** leaf-induced, **b** root-induced and **c** northern blot analyses of TIA pathway genes in leaf-induced CCC, S and R-CCC cultures. Lowest panels show (**a**, **b**) *CrActin* and (**c**) ethidium bromide stained rRNA as controls. For northern analysis, total RNA (20 μg) was separated on 1.5% formaldehyde gel, blotted onto nylon membrane and hybridized to [$\alpha$-$^{32}$P] labeled cDNA of *Str, Tdc*, *G10h* and *Dat*

There was significant induction of the repressor mRNAs in leaf-induced CCC, S and R-CCC cultured cells. Some of these transcripts, *Gbf1, Gbf3, Zct1* were upregulated in suspension cells (Fig. 4a). Similarly, the mRNA levels of *CrBpf1* transcript, which plays a role in elicitor responsive signal transduction pathway of TIA biosynthesis, were high in S and R-CCC cultured cells. Under similar conditions, the mRNA levels of *Orca3*, the master regulator for TIA pathway biosynthesis, were relatively low. However, in root-induced cultures, the expression of this transcript was reverse in S culture (Fig. 4b). The steady state mRNA levels of *Zct1* and *Gbf1* were confirmed by northern hybridization analysis in leaf-induced cultures, but *Orca3* remained undetected (Fig. 4c).

## Analysis of expression profiles of genes and regulators

TIA as well as related primary pathway genes and regulator transcripts in leaf-induced CCC, S and R-CCC culture sets showed a highly differential expression profile as studied using multivariate analysis (Fig. 5a, b). After subsequent data processing, expression values were obtained for each gene. Subsequently, using profile of the relative expression value of each transcript, we performed clustering for all the genes and regulators separately with SYSTAT11 software. Based on their relative abundance, TIA and related primary biosynthetic pathway genes were grouped into two categories, namely, cluster1 (c1) of highly abundant transcripts and cluster2 (c2) of less abundant transcripts. The resulting categories revealed fold changes in the expression level within the rotation culture system. The c1 cluster included five highly abundant primary and early TIA pathway transcripts in CCC, S and R-CCC cultures, except *Omt*. Within, c1, 2 subclusters c1A and c1B were again identified with *Dxr* and *Mecs* in c1A and *Tdc* and *Str* in c1B. Cluster c2 included less abundant three downstream vindoline pathway genes, *T16h*, *D4h* and *Dat*, along with primary pathway genes and were again divided into two sub clusters, c2A and c2B. Within c2A, *Dat* and *Sls* expression profiles synchronized with each other and were further grouped with *D4h*. The remaining genes in c2B showed less to negligible expression and were thus grouped separately (Fig. 5a).

The regulators were separately grouped into two distinct clusters, c3 and c4 based on their high and low abundance revealing a differential expression (Fig. 5b). The highest level of expression was observed in cluster c4, which comprised exclusively of abundant repressors, namely, *Zct1*, Zct2, *Gbf2* and *Gbf3*. Cluster c3, on the other hand included comparatively less abundant repressors, *Zct3* and *Gbf1* along with a transcription factor, *CrBpf1*. The master regulator *Orca3* showing least expression was represented distinctly within subcluster c3 (Fig. 5b).

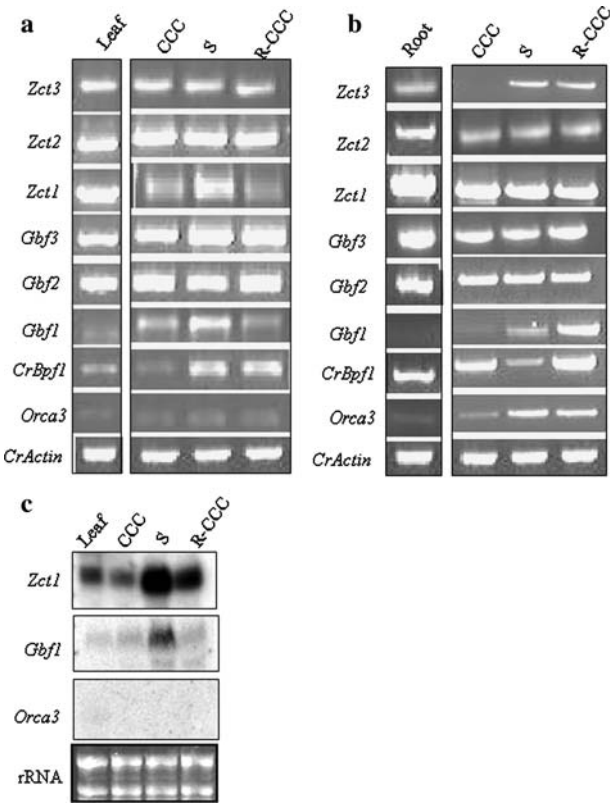


**Fig. 4** Expression patterns of TIA pathway regulators by semiquantative RT-PCR and northern analyses. Semiquantitative RT-PCR analysis of **a** leaf, **b** root-induced and **c** northern analysis of TIA pathway regulators in leaf-induced CCC, S and R-CCC cultures. Lowest panels show (**a**, **b**) *CrActin* and (**c**) ethidium bromide stained rRNA as controls. For northern analysis, total RNA (20 μg) was separated on 1.5% formaldehyde gel, blotted onto nylon membrane and hybridized to [$\alpha$-$^{32}$P] labeled cDNA of *Orca3, Gbf1*, and *Zct1*

## TIAs in *C. roseus* cell cultures

The methanol extracts of leaf-induced CCC, S and R-CCC cultures were analyzed by HPLC (Fig. 6a–c). There were accumulations of two major TIAs, namely, ajmalicine and serpentine and one minor TIA, vindoline in CCC culture. The amounts of these different TIAs were reduced approximately by tenfold in S culture. However, the accumulation of these TIAs was re-initiated upon transfer to R-CCC culture. Thus, threefold more accumulation of ajmalicine and sevenfold to eightfold more accumulation of serpentine were observed in R-CCC as compared to their contents in S

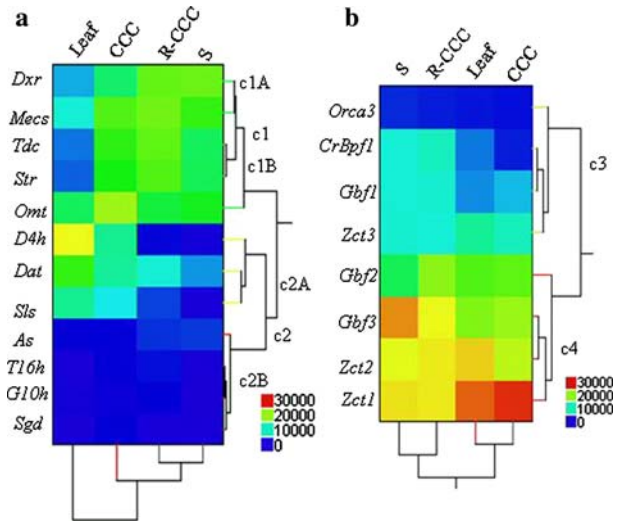


**Fig. 5** Cluster diagram of leaf-induced **a** genes and **b** regulators involved in TIA biosynthesis based on the relative expression level as obtained from RT-PCR data. Grouping was performed using hierarchical clustering, which are represented in four different colors. The intensities of the colors increase with increasing differences in the expression profile of a particular gene as indicated by the side of each figure

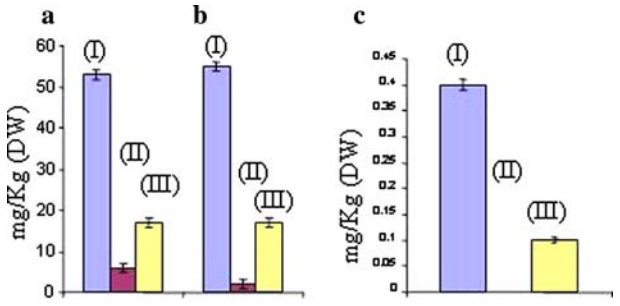


**Fig. 6** HPLC analysis of TIAs in leaf-induced *C. roseus* rotation culture system. The methanol extract of leaf-induced CCC (I), S (II) and R-CCC (III) cultures showed accumulation of **a** ajmalicine, **b** serpentine and **c** vindoline as represented in mg/Kg based on their dry weights (DW). Mean values ± SD obtained for three independent measurements are given

culture (Fig. 6a, b). Accumulation of vindoline was detectable in R-CCC culture but not so in S culture (Fig. 6c). Presence of any other TIAs, namely, catharanthine, vinblastine and vincristine, however remained undetected in *C. roseus* rotation culture system.

## Discussion

Coordinate transcriptional control of biosynthetic genes via transcription factors has been shown to be a major mechanism dictating the final production of secondary metabolites in plants (Endt et al. 2002). In the present study, a number of TIA as well as related primary pathway genes revealed low expression in leaf- as well as root-induced CCC, S and R-CCC cultured cells and a few of others remained undetected at the level of mRNA. On the other hand, steady state high mRNA levels of the repressors, mainly, *Gbf2*, *Gbf3* and *Zct1* were noted in these cultures. A direct relationship of enhanced expression of most of the TIA pathway genes, namely, *G10h*, *Tdc*, *Sls*, *T16h*, *Omt*, *D4h and Dat* was noted with initiation/re-initiation of in vitro differentiation in CCC and R-CCC cultures, respectively. There was a reduction or loss of expression of these genes during cellular proliferation in S culture. Surprisingly, the high expressions of the repressors remain unaffected under similar in vitro cellular conditions in *C. roseus*. So far, ORCA transcription factors have been shown to regulate the MeJA responsive activation of several TIA biosynthetic pathway genes (van der Fits and Memelink 2000; Memelink et al. 2001). Our results demonstrated that TIA pathway repressors might have strong influence in the regulation of many important TIA pathway genes in *C. roseus* cell cultures. This revealed the complexity of regulatory networks and confirmed the close relationship between TIA biosynthetic pathway gene expression and cellular differentiation in *C. roseus*. Accumulation of secondary metabolites in cell culture has often been reported to be influenced by the physical as well as the chemical nature of culture conditions by particularly affecting growth and differentiation of cells in culture, thereby influencing the production of secondary metabolites (Nakagawa et al. 1986). Interestingly, R-CCC showing re-initiation of cellular differentiation showed restoration of a number of alkaloid accumulation which was low in S cultured cells. It is indicative of the fact that the molecular program of cellular differentiation is intimately linked with TIA production. Absence of a few TIAs in *C. roseus* rotation culture system in the present case confirms the earlier reports of inability of cell cultures to produce a number of important TIAs (Vazquez-Flota et al. 2002).

In conclusion, a combined transcript profiling of TIA pathway genes, activator and repressors has been shown for the first time in *C. roseus* cell cultures, which revealed highly abundant TIA pathway repressor transcripts in CCC, S and R-CCC cultures along with a few important genes and their activators. Thus, a few other important TIA biosynthetic pathway genes were represented by lower levels of expression in *C. roseus* cell cultures in different culture conditions while some other genes were not expressed at all. Hence, in order

to utilize *C. roseus* cell suspension system as an alternate source of TIA production, a fine tuning of the induction of TIA pathway repressors and activators is necessary. Such a system is assumed to act via switch on/off of the positive and negative regulators in such a way that it finally leads to the enhanced expression of important TIA biosynthetic pathway genes leading to improved production of final TIAs in *C. roseus* cell culture. On the other hand, suppression of repressors may also be an alternative viable system for improved production of TIAs in cell culture, which has not yet been achieved.

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