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ORIGINAL ARTICLE

Overexpression of tryptophan decarboxylase and strictosidine synthase enhanced terpenoid indole alkaloid pathway activity and antineoplastic vinblastine biosynthesis in *Catharanthus roseus*

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Abstract

Terpenoid indole alkaloid (TIA) biosynthetic pathway of *Catharanthus roseus* possesses the major attention in current metabolic engineering efforts being the sole source of highly expensive antineoplastic molecules vinblastine and vincristine. The entire TIA pathway is fairly known at biochemical and genetic levels except the pathway steps leading to biosynthesis of catharanthine and tabersonine. To increase the in-planta yield of these antineoplastic metabolites for the pharmaceutical and drug industry, extensive plant tissue culture-based studies were performed to provide alternative production systems. However, the strict spatiotemporal developmental regulation of TIA biosynthesis has restricted the utility of these cultures for large-scale production. Therefore, the present study was performed to enhance the metabolic flux of TIA pathway towards the biosynthesis of vinblastine by overexpressing two upstream TIA pathway genes, tryptophan decarboxylase (*CrTDC*) and strictosidine synthase (*CrSTR*), at whole plant levels in *C. roseus*. Whole plant transgenic of *C. roseus* was developed using *Agrobacterium tumefaciens LBA1119* strain having *CrTDC* and *CrSTR* gene cassette. Developed transgenic lines demonstrated up to twofold enhanced total alkaloid production with maximum ninefold increase in vindoline and catharanthine, and fivefold increased vinblastine production. These lines recorded a maximum of 38-fold and 65-fold enhanced transcript levels of *CrTDC* and *CrSTR* genes, respectively.

Keywords *Catharanthus roseus* · Terpenoid indole alkaloids (TIAs) · Tryptophan decarboxylase (*CrTDC*) · Strictosidine synthase (*CrSTR*) · Sonication-assisted *Agrobacterium*-medicated transformation (SAAT)

Introduction

Catharanthus roseus (L.) G. Don. (Family Apocynaceae) is a highly important medicinal plant with immense medicinal value because of its potential to synthesize more than 130 known bioactive terpenoid indole alkaloids (TIAs) (El-Sayed and Verpoorte 2007; Facchini and De Luca 2008; Verma et al. 2012). *C. roseus* gained classical medicinal values for being the sole source of the two most valuable antineoplastic alkaloids vinblastine and vincristine and also produces two antihypertensive alkaloids ajmalicine and serpentine (van der Heijden et al. 2004; Verma et al. 2015a, b). The low *in-planta* production and unavailability of synthetic alternate have amplified the high market demand and exorbitant price of antineoplastic drug molecules vinblastine and vincristine. These limitations have brought *C. roseus* to the center stage of terpenoid indole alkaloid biosynthetic pathway of metabolic engineering efforts in recent years (Guirimand et al. 2011a, b; Wang et al. 2012, 2016; Pan et al. 2012; Liu et al. 2017). The extensive knowledge gained over the past four decades suggested that biosynthesis of TIAs in *C. roseus* is highly complex and strictly regulated through developmental, environmental, and organo- and cell-specific controls and operates through a coordinately regulated network of more than 30 enzymatic steps and 35 known intermediate molecules

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(Rischer et al. 2006; Verma et al. 2012; Liu et al. 2017). Therefore, the greatest challenge for the researchers is to explore the technologies to enhance the in vitro production of antineoplastic drug molecules and their precursors in *C. roseus*. This technological gap has established *C. roseus* as a well-recognized model system to study the regulation and biosynthesis of terpenoid indole alkaloids (Zhao and Verpoorte 2007).

The dynamics and hierarchy of TIA biosynthetic pathway assembly have evidently suggested that TIA biosynthesis pathway is far more than a straightforward linear arrangement of consecutive enzymatic reactions of indole, (MEP), and secoiridoid pathway (Pasquali et al. 2006; Facchini and De Luca 2008; Verma et al. 2012). This special model of TIA pathway requires four different cellular organizations (epidermis, laticifers, idioblasts, and internal phloem-associated parenchyma) and five intracellular compartments (chloroplast, cytosol, nucleus, vacuole, and endoplasmic reticulum) for the production of dimeric alkaloids vinblastine and vincristine (Verma et al. 2012; Wang et al. 2012; Pan et al. 2016). The cellular and subcellular organization of TIA biosynthetic pathway also presents a very interesting picture that suggests the necessity of inter-organelle trafficking of pathway intermediates (Facchini and St-Pierre 2005; Mahroug et al. 2007; Liu et al. 2017).

Geraniol is synthesized by MEP pathway inside the chloroplast of internal phloem-associated parenchyma cell of leaf, whereas shikimate pathway produces the aromatic amino acid tryptophan (Mahroug et al. 2007). Tryptophan is converted to tryptamine by the activity of enzyme tryptophan decarboxylase (TDC) in the cytoplasm (Fig. 1 (i)) (De Luca and Cutler 1987). Geraniol is converted into secologenin via a series of enzymatic conversation through enzymes present in endoplasmic reticulum (Miettinen et al. 2014). Now both tryptamine and secologanin are transported to vacuoles (Guirimand et al. 2009). TIA

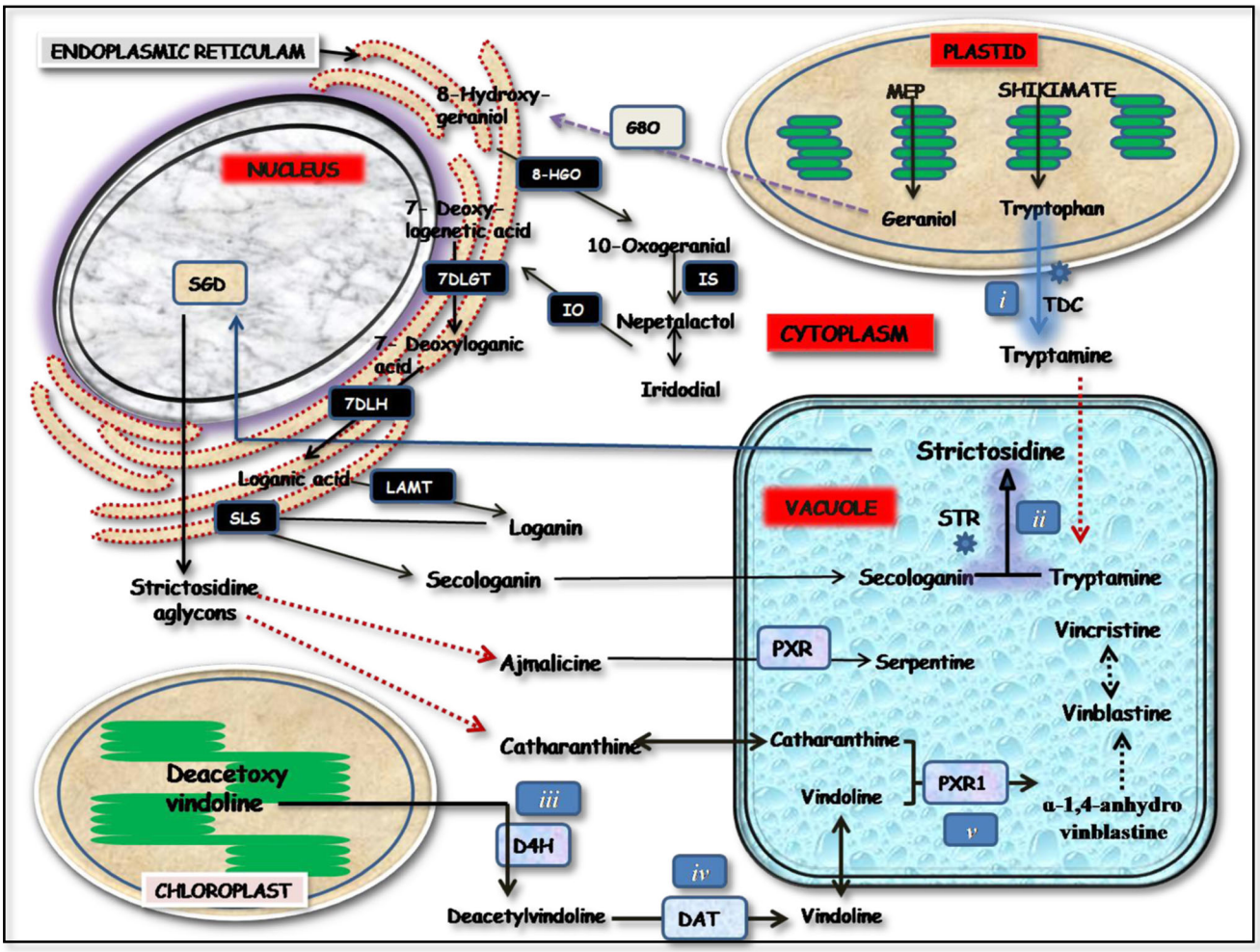


Fig. 1 Schematic representation of terpenoid indole alkaloid pathway in *Catharanthus roseus*. *G8H geraniol 8-hydroxylase*; *8HGO 8-hydroxygeraniol oxidoreductase*; *IS iridoid synthase*; *IO iridoid oxidase*; *7DLGT 7-deoxyloganic acid glucosyltransferase*; *7DLH 7-deoxyloganic acid hydroxylase*; *LAMT loganic acid methyltransferase*; *SLS secologanin synthase*; *D4H desaetoxyvindoline-4-hydroxylase*; *DAT deacetyl-vindoline-4-O-acetyltrasnferase*; *TDC tryptophan decarboxylase*; *STR strictosidine synthase*; *SLS secologanin synthase*; *SGD strictosidine-β-D-glucosidase*; *PXR peroxidase. i, ii, iii, iv, v*: different enzymatic reaction; ⟶ molecule transportation; ⇢ series of enzymatic reaction

biosynthesis starts with the condensation of tryptamine and secologanin to form the central intermediate of TIA pathway, strictosidine by strictosidine synthase (STR) (Fig. 1 (ii)) (McKnight et al. 1991; Oudin et al. 2007). This step indicates the switchover of primary metabolism to secondary metabolism during TIA biosynthesis. Strictosidine is further processed by enzyme strictosidine-β-glucosidase (SGD) and produces highly reactive strictosidine aglycone (Stevens et al. 1993). This strictosidine aglycone undergoes various structural and molecular reorganizations and produces different molecular skeletons to yield different TIAs (Verpoorte et al. 1997; Geerlings et al. 2000). Two major enzymatic conversions in strictosidine aglycone lead to the production of (a) ajmalicine and serpentine via cathenamine and (b) vindoline and catharanthine via stemmadenine through multiple steps in different cellular and subcellular components. The formation of ajmalicine and serpentine is very well documented. However, few genes, enzymes, or intermediates involved in the vindoline biosynthesis from cathenamine to tabersonine have not yet been characterized, whereas, the catharanthine biosynthesis is yet to be discovered (Liu et al. 2017). Finally, monomeric alkaloids catharanthine and vindoline dimerized to produce a-3′,4′-anhydrovinblastine (AVLB), by the activity of peroxidase a-3′,4′-anhydrovinblastine synthase (PRX1) (Fig. 1 (v)) (Costa et al. 2008). Finally, vinblastine and vincristine are now consequentially derived from a-3′,4′-anhydrovinblastine through several steps that have yet not been characterized (Guirimand et al. 2010; Verma et al. 2012).

Being the sole source of natural antineoplastic drug molecules, biosynthesis of TIAs has been extensively studied in *C. roseus*. The studies were focused in the direction to understand the genetic and developmental regulation of TIA biosynthesis and accumulation with an aspire to increase their production in in vitro cell, tissue, and organ cultures using the approaches of metabolic engineering. The cell culture-based in vitro production of secondary metabolites mainly requires (i) an adequate supply of precursors, (ii) optimal induction enzymes and gene biosynthetic pathway, and (iii) availability of enough sink to store the synthesized metabolites (Mathur et al. 2006; Tyo et al. 2007). In metabolic engineering-based approach, overexpression of inherent pathway gene(s) in the native plant system is considered as the favorable alternate way to enhance the whole metabolic pathway flux towards the accumulation of desired metabolite(s) (Pan et al. 2012; Miralpeix et al. 2013; Mishra et al. 2016). The TIA pathway modulation efforts in *C. roseus* are basically aimed towards either pushing or pulling the pathway metabolic flux towards the desired products by up- or down-regulating the TIA pathway genes. Utilizing the cell culture-based metabolic engineering approaches, many genes like geraniol 8-hydroxylase (*CrG8H*), 1-deoxy-D-xylulose synthase (*CrDXS*), tryptophan decarboxylase (*CrTDC*), secologanin synthase (*CrSLS*), strictosidine synthase (*CrSTR*), strictosidine beta-glucosidase (*CrSGD*), desacetoxyvindoline 4-hydroxylase (*CrD4H*), deacetylvindoline-4-O-acetyltransferase (*CrDAT*), apoplastic peroxidase (*CrPRX1*), anthranilate synthase (*CrAS*), and transcription factors ORCA2, ORCA3, BIS1, and BIS2 were cloned and overexpressed in in vitro cell suspensions and hairy root cultures of *C. roseus* (Canel et al. 1998; Hughes et al. 2004a, b; Magnotta et al. 2007; Peebles et al. 2011; Liu et al. 2011; Kumar et al. 2011; van Moerkercke et al. 2015). The results obtained from these studies have shown that depending upon their level of cellular organization, morphological differentiation, and specific culture conditions, cell suspensions and hairy root cultures are competent for synthesizing only the monomeric TIAs like ajmalicine, catharanthine, serpentine, and vindoline but failed to synthesize dimeric TIAs vinblastine and vincristine due to their lesser degree of cellular/tissue differentiation. The synthesis of dimeric TIAs vinblastine and vincristine is specifically limited to the leaves (the actual site of TIA biosynthesis) (Mahroug et al. 2007; Verma and Mathur 2011a). This major gap and limitation in *C. roseus* biotechnology have been generally ascribed to its genetically non-tractable and recalcitrant nature for in vitro plant regeneration via adventitious routes (Facchini and De Luca 2008; Verma et al. 2012). Genetic engineering at whole plant level for TIA pathway modulation in *C. roseus* is, therefore, a technological gap that needs to be bridged. In metabolic engineering, a single-step approach (single-gene overexpression) has the limitation of thorough knowledge of rate-limiting steps in the metabolic pathway which can be overcome by multi-step engineering of a metabolic pathway by expressing the gene-encoding enzymes that act upon different biosynthetic steps (Verpoorte and Alfermann 2000; Twyman et al. 2003). This technique can be used as major metabolic engineering approach intended to enhance the metabolic flux towards the desired metabolite (Hughes and Shanks 2002). Recently, genetic transformation of *C. roseus* was also performed to obtain transgenic plants overexpressing *CrDAT* (Wang et al. 2012), *CrG10H*, and *CrORCA3* genes (Pan et al. 2012).

The present work intended to fill this gap by overexpressing two genes upstream of the TIA pathway, *CrTDC* and *CrSTR*, at whole plant level in *C. roseus* to investigate their effect on TIA biosynthesis. Changes in the transcripts of important TIA pathway genes *CrTDC*, *CrSTR*, *CrSGD*, *CrT3O*, *CrT3R*, *CrDAT*, *CrD4H*, and *CrPRX1*, along with the accumulation of monomeric TIAs vindoline and catharanthine and dimeric TIAs vinblastine and vincristine, were analyzed to understand the effect of overexpression of *CrTDC* and *CrSTR* on TIA biosynthesis.

Material and methods

Plant material

The explants required for regeneration and transformation experiments were obtained from the in vitro grown multiple shoot cultures of *C. roseus cv.* Dhawal [National Gene Bank

(CIMAP) accession no. 0859] maintained on a shoot multiplication medium (SMM) constituted of MS medium (Murashige and Skoog 1962) fortified with 1.0 mg L^{-1} 6-benzylaminopurine (BAP; Sigma-Aldrich®, St. Louis, MO), 0.1 mg L^{-1} naphthalene acetic acid (NAA; Sigma-Aldrich®), 0.40 mgL^{-1} thymine hydrochloride (THCL; Sigma-Aldrich®), 30 g L^{-1} sucrose (Hi-Media Laboratories Pvt. Ltd., India), and 4 g L^{-1} Phytagel (Sigma-Aldrich®) (Verma and Mathur 2011b). All cultures were maintained under the photoperiod of 16 h using white fluorescent tubes (48 μmol m^{-2} s^{-1}, Philips) at 26 ± 2 °C temperature. Leaves of 1-month old in vitro grown multiple shoot cultures were used as the experimental material for subsequent transformation studies.

Bacterial strain and vector used for transformation

Transformation work was performed with the *Agrobacterium tumefaciens* LBA1119 strain integrated with the binary vector pMOG22 (Mogen, Leiden, The Netherlands) having *CrTDC and CrSTR* gene cassette (<*hpt*-<*Tdc2*-<*Str-gus*>) (Fig. 2; a kind present of Prof. Johan Memelink, University of Leiden, The Netherlands, earlier used in Verma et al. 2015a; Sharma et al. 2017a, b). The *CrTDC and CrSTR* gene construct encodes resistance to hygromycin as plant selection marker and a GUS reporter gene (with interrupted coding sequences using an intron) (Vancanneyt et al. 1990). *CrTDC and CrSTR* gene cassette used *CaMV 35S* promoter with double enhancer region and *Nos* terminator sequences. Bacterial culture of *A. tumefaciens* was cultured in 50-mL LC medium (Canel et al. 1998) having 10.0 g L^{-1} tryptone, 8.0 g L^{-1} NaCl, and 5.0 g L^{-1} yeast extract, added with 50 mg L^{-1} rifampicin and 100 mg L^{-1} kanamycin, using a single bacterial colony at 28 °C under dark conditions on a gyratory shaker (200 rpm).

Genetic transformation

To develop transgenic shoot buds, the healthy growing 30-day-old leaf explants were pre-plasmolyzed in 13% (*w*/*v*) mannitol (Hi-Media) for 30 min and incubated onto a shoot bud regeneration medium (SBM) containing WP medium (Woody Plant Medium, Lloyd and McCown 1980) + 4.5 mg L^{-1} BAP + 2.5 mg L^{-1} NAA (modified after Verma and Mathur 2011b) for 10 days. After the incubation period, the leaves were subjected to sonication-assisted *Agrobacterium* transformation (SAAT) (Trick and Finer 1997). During the SAAT, leaves were dipped in a 36-h grown bacterial culture (0.48 OD_{600}) and kept in an ultrasonic bath for 30 s (30 ± 3 kHz/250 W, Ultrasonic Cleaner, Rivotek™) and co-cultivated over SBM for 48 h. Due to the unavailability of *A. tumefaciens* LBA1119 strain devoid of empty vector pMOG22, the non-transformed tissues were used as a control.

Plant regeneration and selection of putative transformants

After co-cultivation, transformed leaves were shifted to SBM fortified with 500 mg L^{-1} cefotaxime and 100 mg L^{-1} vancomycin for bacterial clearance and then to antibiotic-free SBM

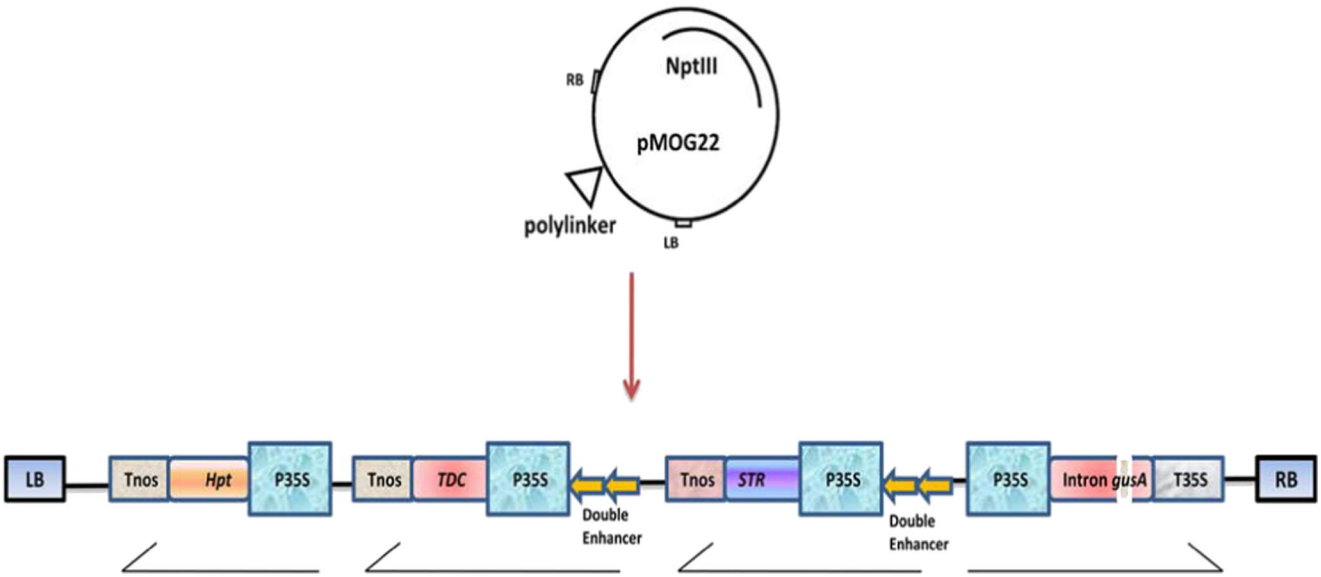


Fig. 2 T-DNA region of *CrTDC* + *CrSTR* gene construct used in the present study. *Tnos nopaline synthase terminator*; *hptII hygromycin phosphotransferase gene*; *P35S-CaMV cauliflower mosaic virus 35S promoter*; *T35S-CaMV cauliflower mosaic virus 35S terminator*; *gusA intron-β-glucuronidase gene containing intron*; *RB right border*; *LB left border of T-DNA*; ⟍ *direction of transcription. (*Canel et al. 1998; *Courtesy Johan Memelink)*

for shoot bud regeneration. The regenerated shoot buds were subsequently plated over the selection medium (SBM + 20mg L^{-1} hygromycin + 500 mg L^{-1} cefotaxime) for the development of putatively transformed shoots. The regenerated shoots survived over selection medium were then transferred onto the half strength MS medium devoid of plant growth regulators for shoot elongation. The roots were developed over rooting medium (RM) consisted of MS medium fortified with 3.0 mg L^{-1} Indole-3-butyric acid (IBA). The transformed shoots were maintained in vitro, and rooted plants were hardened and acclimatized successfully in an autoclaved mixture of an equal proportion of garden soil, sand, and vermiculite in pots under the normal glasshouse conditions of 28 ± 4 °C temperature and 60–70% RH for further analysis.

Histological studies

To trace the shoot bud induction in incubating transformed leaf, the tissue was fixed in a 70% ethanol to acetic acid to formalin ratio (18:1:1 *v/v*), dehydrated through a graded ethanol-butyl alcohol series, and embedded in paraffin wax (Johanson 1940). Serial transverse sections (10–15 μm) were cut on a rotary microtome, deparaffinized in a xylene series, stained with safranine (1% *w/v*), and mounted in Canada balsam for microscopic examination.

GUS assay

Professedly transformed shoots survived over selection medium were analyzed for histochemical analysis of GUS *uidA* gene expression as per the procedure of Jefferson et al. (1987). In brief, the transformed tissues were engrossed overnight at 37 °C in an assay mixture containing 1 mM X-gluc (Sigma-Aldrich®), 50 mM sodium phosphate buffer (pH 7.0), and 0.1% (*v/v*) Triton X-100 (Hi-Media) for GUS staining. Stained transformed tissues were washed in 70% (*v/v*) ethanol (Merck Specialities Pvt. Ltd., Mumbai, India) at 28 ± 2 °C temperature to remove the remaining chlorophyll until the blue color became evident. Histochemical analysis and photography were performed using a binocular microscope (S8-APO, Leica).

Molecular analysis of putative transformants

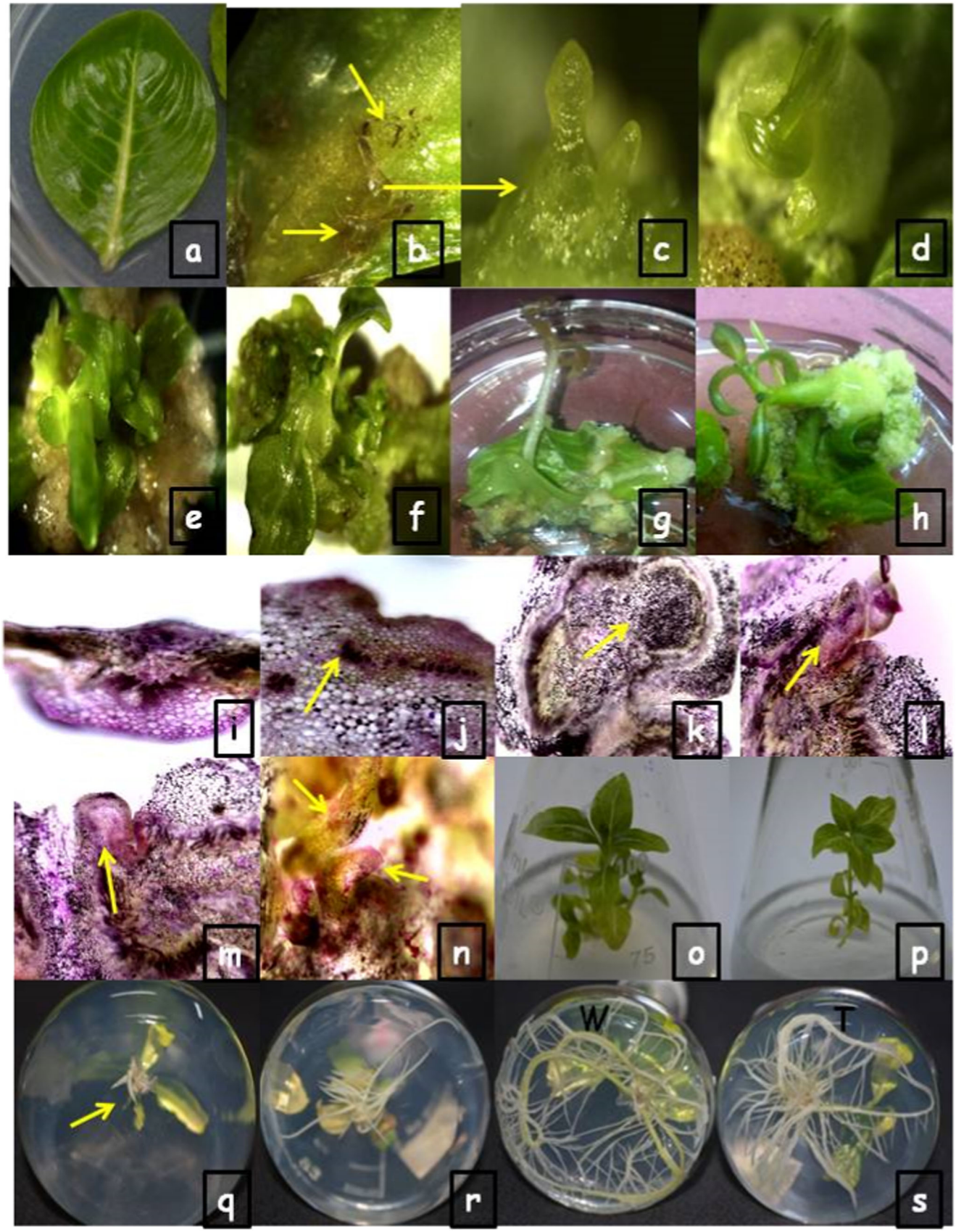
The PCR-based detection of GUS *uidA* and hygromycin *hptII* genes were performed in the GUS-positive shoots. The transformed and non-transformed tissues were subjected to genomic DNA isolation using cetyl trimethyl ammonium bromide (CTAB) method. A 25-μL reaction mixture of PCR contained DNA template (20 ng), 2.5 μL Taq buffer (10X), 1 μL dNTPs mix (100 mM), *hptII*- and *uidA*-specific forward and reverse primers (10 pmol; 1 μL), and Taq DNA polymerase (1.25 unit). The PCR thermocycling parameters remain: single cycle of initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation for 1 min at 94 °C, primer annealing for 1 min at 55 °C, and extension for 2 min at 72 °C and a single cycle of final extension at 72 °C for 5 min. The PCR products were alienated on 1.2% (*w/v*) agarose gel (Sigma-Aldrich®), stained with Nancy-520 (Sigma-Aldrich®), and observed under UV (300 nm) using a Gel Documentation system (ProteinSimple). Southern blotting analysis was performed using protocol earlier described in Sharma et al. (2017a). Briefly, genomic DNA (50 μg) of transgenic and non-transgenic control plants was digested with *AgeI* (for *CrTDC*)/*BsmI* (for *CrSTR*) and separated by electrophoresis over 0.8% (*w/v*) agarose gel. Bands separated on gel were transferred to a positively charged Hybond N+ nylon membrane (GE Healthcare) and hybridized with digoxigenin-labeled *CrTDC* and *CrSTR* probes. Probe synthesis and signal detection were performed using DIG DNA Labelling and Detection Kit (Roche Life Sciences, USA).

Real-time quantitative PCR analysis

To analyze the quantitative real-time PCR the expression of transgenes, total RNA was extracted from the transformed and non-transformed shoots according to the manufacturer's instructions (DSS Takara) and treated with DNase I (Thermo Scientific). The purity and quantity of extracted RNA were determined using NanoDrop spectrophotometer (Thermo Scientific, USA). The first-strand complementary DNA (cDNA) from the RNA was generated using the random primers, using the MultiScribe Reverse Transcriptase kit (Applied Biosystems, USA). The qRT-PCR program consisted of an initial hold at 50 °C for 2- and 10-min incubation at 95 °C followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Relative transcript expression of eight candidate genes viz., *CrTDC*, *CrSTR*, *CrSGD*, *CrT3O*, *CrT3R*, *CrDAT*, *CrD4H*, and *CrPRX1*, was determined using the Livak's $2^{-\Delta\Delta Ct}$ method. The threshold cycle (*Ct*) for each gene was normalized against the *Ct* for actin of *C. roseus*, which was previously used as the endogenous gene (Verma et al. 2015a; Pandey et al. 2016; Kumar and Bhatia 2016). List of primers used in the present study is provided in Supplementary Material.

Metabolite profiling and quantification

For alkaloid extraction, 100-mg oven-dried (50–60 °C) leaf samples from transformed shoots were extracted thrice using methanol (HPLC grade; 3 × 30 mL) at 28 ± 2 °C temperature. These methanolic extracts were collected and dehydrated under vacuum to 10 ml, and 10-mL distilled water was added. The mixture was acidified with 10 mL of 3% (*v/v*) HCl and washed thrice with hexane (3 × 30 mL). The purified aqueous portion was basified with ammonia (to pH 8.0), extracted with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◀ **Fig. 3** Different stages of transgenic shoot production. **a** Leaf explant co-cultivated with *A. tumefaciens* LBA1119. **b–d** Shoot bud induction after 30 days of co-cultivation over SBM. **e**, **f** Shoot bud elongation after 40 days and **g**, **h** 50 days over SBM. **i–n** Microscopic view of shoot bud induction (stained with 1% safranine), **o–p** established transgenic shoot over SMM. **q** Root induction, **r** elongation, and **s** complete rooting over RM. W wild; T transgenic; SBM WPM + 4.5 mg L^{-1} BAP + 2.5 mgL^{-1} NAA; SMM MS + 1.0 mg L^{-1} BAP + 0.1 mg L^{-1} NAA + 0.1 mg L^{-1} THCL; RM MS + 3.0 mg L^{-1} IBA. Arrow indicates the point of origin

chloroform (3 × 30 mL), air-dried over anhydrous sodium sulfate, and weighed until constant reading. The total alkaloid extracts obtained were analyzed through high-performance liquid chromatography (HPLC) for determination of monomeric alkaloids vindoline, catharanthine, ajmalicine, and serpentine and dimeric alkaloids vinblastine and vincristine. The standards were purchased from Sigma-Aldrich, USA. Waters LV-8A gradient HPLC system and RP-18e reverse-phased chromolith performance HPLC column were used for HPLC analysis. The HPLC system was fitted with LC-8A pumps, SPD-M10*Avp* PDA detector, and manual injector valve. Mobile phase used was made up of acetonitrile: ammonium acetate (100 mM, pH 7.3). Detection was done at 254 nm. Availability of tryptophan in the free amino acid pool was determined using colorimetric estimation; secologanin and tryptamine quantification in transformed and non-transformed tissues was performed using HPLC as per the protocols earlier described in Verma et al. (2014) and Sharma et al. (2017a, b), respectively.

Statistical analysis

The experimental data obtained in the present study is expressed as the mean ± standard deviation (SD) of three independent biological replicates. The statistical differences between total alkaloid contents, vindoline, catharanthine, vinblastine, and free tryptophan, and tryptamine content were analyzed using two-way analysis of variance (ANOVA) using SPSS (version 17.0). Values at $P < 0.01$ were considered statistically significant. To check the significance of the experiment, results in the post hoc Tukey's test and bivariate, two-tailed, Pearson's correlation analysis between the above metabolic content were performed using the SPSS software package version 17.0 (SPSS Inc., USA).

Results

Agrobacterium-mediated transformation of *C. roseus*

In order to assess the possibility of regenerating stable whole plant transformants with *CrTDC* and *CrSTR* gene integration, the transgenic shoot bud regeneration protocol was employed. For this, the pre-plasmolyzed leaf explants were initially incubated over SBM for 10 days before subjecting to SAAT with *A. tumefaciens* strain LBA1119 harboring *CrTDC* and *CrSTR* gene cassette. Post SAAT, leaves were co-cultivated for 48 h on SBM and shifted to fresh SBM containing 500 mg L^{-1} cefotaxime and 100 mg L^{-1} vancomycin to kill the bacterial population. Later, the co-cultivated explants were shifted to antibiotic-free SBM for shoot bud organogenesis (Fig. 3a) that became evident after 25–30 days incubation (Fig. 3b–d) and attended the height of 3–4 cm in 40–50 days (Fig. 3e–h). The light microscopic examination of transverse sections of responded leaf explants on SBM revealed increased meristematic activity around midrib zone within 8–10 days of culturing (Fig. 3i–k). The organized shoot primordia started appearing in the form of protuberances from hypodermal tissues and developed into well-defined shoot buds (Fig. 3l–n). The absence of intervening callus and continuity of vascular tissue of regenerated buds with parent tissue confirmed their direct mode of shoot bud regeneration. After 40–50 days of incubation, the shoot buds with 3–4-cm height were detached from parent leaf explant and transferred onto a selection medium (SBM fortified with 20 mg L^{-1} hygromycin and 500 mg L^{-1} cefotaxime) for 2 weeks for the recovery of putatively transformed shoots. The shoots that survived over the selection medium were shifted to half strength MS basal medium for 2 weeks and then transferred to SMM medium for multiple shoot formation and growth (Fig. 3o, p). Rooting of the transformed shoots was achieved on the tenth day after rearing them on RM medium (Fig. 3q–s).

A total of six putative transformed shoots were finally recovered with 0.12 recovery rate (6 transformed shoots from 50 transformed leaves) from which four showed clearly visualized GUS staining (Fig. 4a–f). These GUS-positive shoots were subjected to PCR amplification of *uidA* and *hptII* genes. Amplified fragments of *uidA* (366 bp) and *hptII* (800 bp) were observed in all four GUS stained shoots (Fig. 4g, h). Southern blot analysis performed to check the integration of extra copies of *CrTDC* and *CrSTR* genes in all four hygromycin-resistant and GUS-positive transgenic shoots confirmed the insertion of one extra integrated copy of the construct in the transformed plants compared to non-transformed control (Fig. 4i, j). The position of hybridization signals of integrated copies varied in all transgenic lines suggested the occurrence of independent transformation events in the transgenic plant production.

Effect of *CrTDC* and *CrSTR* gene overexpression on tryptamine accumulation and TIA pathway activity

A maximum of a twofold increase in the total alkaloid content was recorded in transgenic line AS3 in comparison to non-transformed control plant followed by in AS4, AS2, and AS1 lines. Secologanin accumulation was estimated marginally

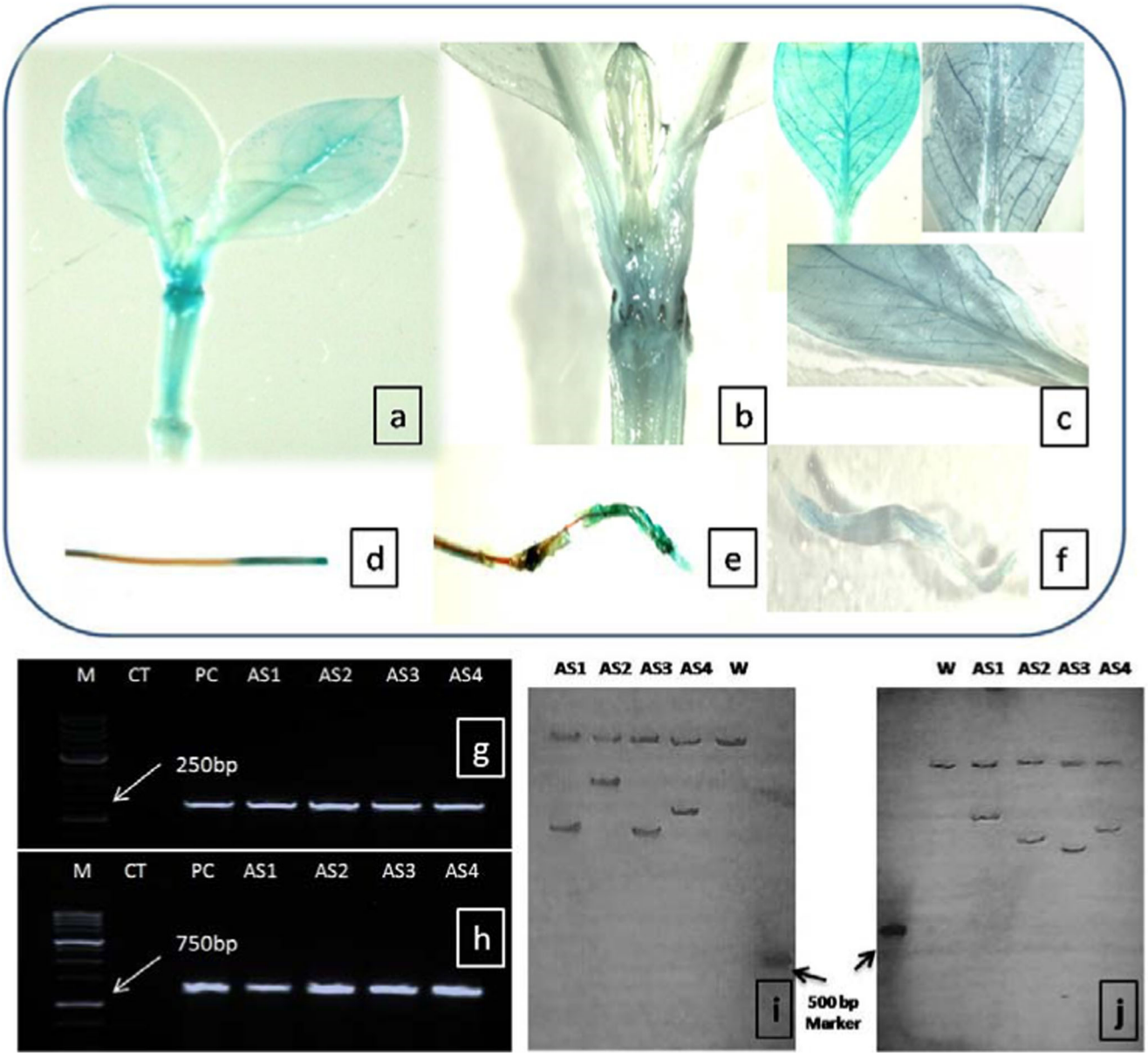

Fig. 4 GUS expression assay of transformed plant: **a** transformed shoot, **b** internode, **c** leaves; **d** stem, and **e**, **f** roots. PCR amplified bands of **g** *uidA* gene (366 bp) and **h** *hpt II* gene (800 bp) in transformed leaves. Southern blot of **i** *CrTDC* and **j** *CrSTR* genes. M marker, CT/W control/wild, PC plasmid control, AS1–AS4 samples

enhanced (90.1–103.7 μg g^{-1} dry wt) when compared to control (89.7 μg g^{-1} dry wt) (Table 1). HPLC analysis revealed that transgenic line AS3 accumulated highest ninefold increased vindoline and catharanthine with a fivefold increase in vinblastine content. Transgenic lines AS2, AS4, and AS1 also demonstrated up to sevenfold increase in vindoline, up to sixfold increase in catharanthine, and up to a fourfold increase in vinblastine content (Table 1). However, ajmalicine, serpentine, and vincristine were not detected in any transformed and non-transformed plant. The qRT-PCR gene expression analysis of transgenic lines reported a maximum of 65-fold increase in *CrSTR* expression with a 38-fold increase in *CrTDC* expression in transgenic line AS3 along with a 21-fold increase in *CrSGD*, up to threefold *CrT3O*, up to twofold *CrT3R*, ninefold increase in *CrD4H*, and a sevenfold increase in *CrDAT* with more than a sevenfold increase in *CrPRX1* (Fig. 5). Transgenic line AS2 exhibited more than 42-fold *CrSTR* and 19-fold increase in *CrTDC* expression. AS2 also displayed a 13-fold *CrSGD*, more than twofold *CrT3O*, up to twofold *CrT3R*, sevenfold *CrD4H*, fourfold *CrDAT*, and a sixfold increase in *CrPRX1* transcript expression. Transgenic lines AS1 and AS4 also expressed relatively increased levels of all genes studied when compared to non-transformed control plant.

All transgenic lines showed marginally less (413–462 μg g^{-1} dry wt) tryptophan content when compared to non-transformed control plant (480.1 μg g^{-1} dry wt)

Table 1 Tryptophan, secologanin, and alkaloid contents in transformed and non-transformed control *C. roseus* plants

Sample	Total alkaloid content (% dry wt ± SD)	Vindoline (% dry wt ± SD)	Catharanthine (% dry wt ± SD)	Vinblastine (% dry wt ± SD)	Tryptophan (μg/g dry wt ± SD)	Secologanin (μg/g dry wt ± SD)	Tryptamine (μg/g dry wt ± SD)
AS1	1.2 ± 0.11	0.17 ± 0.03	0.10 ± 0.02	0.011 ± 0.002	413.2 ± 31.1	96.1 ± 5.7	280.9 ± 123.3
AS2	1.4 ± 0.13	0.29 ± 0.04	0.18 ± 0.03	0.013 ± 0.002	425.4 ± 33.3	101.1 ± 9.1	212.8 ± 126.8
AS3	1.9 ± 0.15	0.47 ± 0.06	0.26 ± 0.04	0.014 ± 0.003	441.3 ± 35.1	103.7 ± 9.7	190.4 ± 132.8
AS4	1.7 ± 0.12	0.26 ± 0.04	0.18 ± 0.03	0.012 ± 0.002	462.4 ± 35.3	90.8 ± 7.2*	248.6 ± 138.6
Control	1.0 ± 0.08	0.04 ± 0.01	0.03 ± 0.01	0.003 ± 0.001	480.1 ± 36.4	89.7 ± 5.7	64.4 ± 15.8

(Data expressed as the mean ± standard deviation (SD) of three independent biological replicates with three technical replicates for each biological replicate)

*Values are not different to control at $P < 0.01$

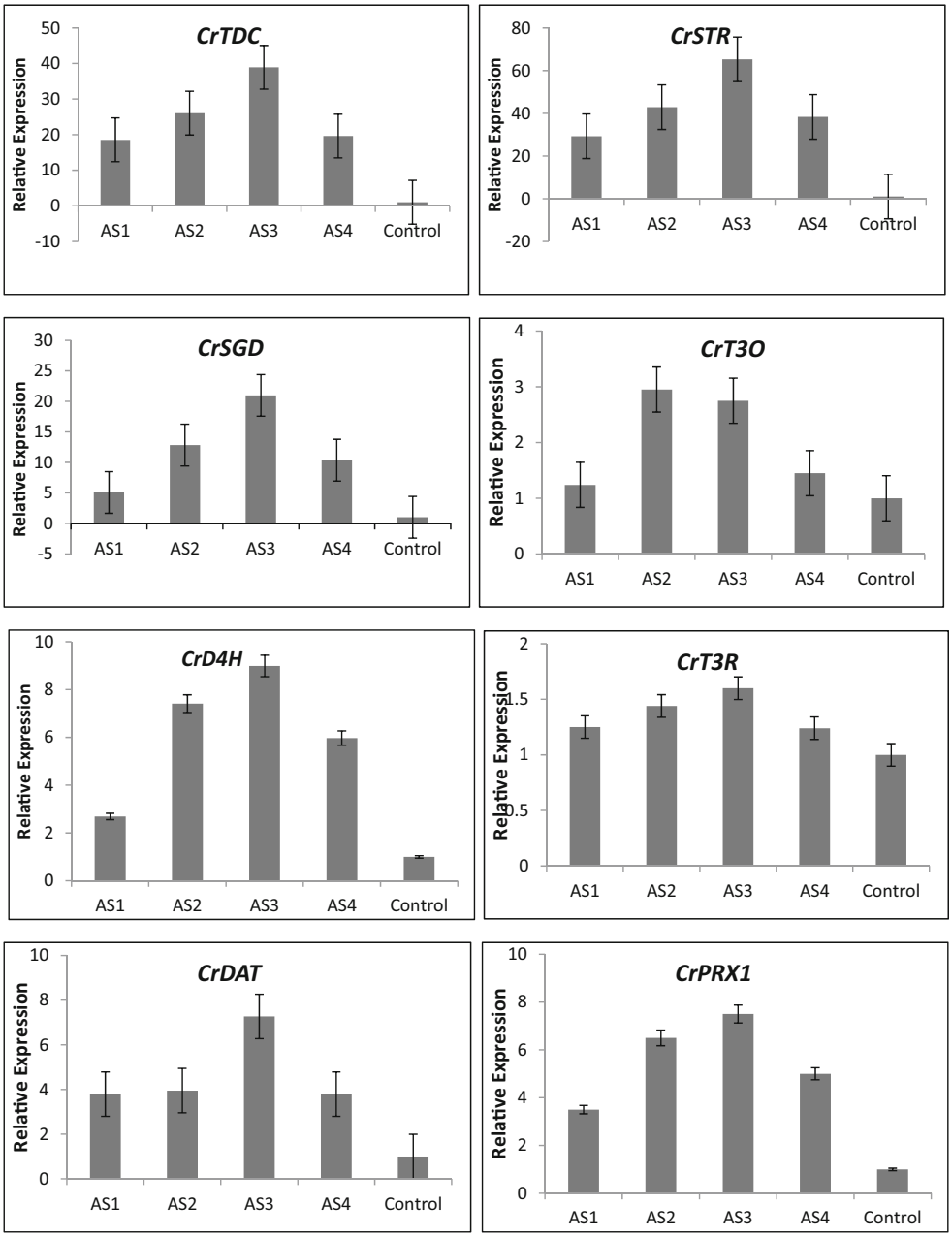

Fig. 5 Relative quantification of TIA pathway genes in *CrTDC* and *CrSTR* overexpressing transgenic plants (AS1-AS4) and wild-type control (CNT). (Data expressed as the mean ± standard deviation (SD) of three independent biological replicates with three technical replicates for each biological replicate; relative expression: Fold increase in gene expression levels compared to control.) STR strictosidine synthase, TDC tryptophan decarboxylase, SGD strictosidine beta-D-glucosidase, T3O tabersonine 3-reductase, T3O tabersonine 3-oxidase, D4H deacetoxyvindoline 4-hydroxylase, DAT deacetylvindoline-4-O-acetyltransferase, *PRX peroxidase1*. Significance $P < 0.05$

(Table 1). The functional analysis of *CrTDC* activity was estimated by analyzing the synthesis of tryptamine from tryptophan. HPLC analysis revealed the highest tryptamine accumulation in transgenic line AS1 (280.9 μg g^{-1} dry wt) followed by AS4, AS2, and AS3 (ranging from 248.6 to 190.4 μg g^{-1} dry wt), compared to non-transformed control plant (64.4 μg g^{-1} dry wt). Transgenic line AS3 with highest *CrTDC* expression (38-fold) levels reported lower tryptophan (441.3 μg g^{-1} dry wt) and tryptamine (190.4 μg g^{-1} dry wt) contents. The same pattern was also followed by transgenic lines AS2, AS4, and AS1, where high *CrTDC* levels were associated with lower tryptophan but "higher" tryptamine levels than the transformed control plants. In general, the transgenic lines accumulate less tryptophan and more tryptamine than the control plants. Non-transformed control plant reported highest tryptophan (480.1 μg g^{-1} dry wt) content with lowest levels of tryptamine (64.4 μg g^{-1} dry wt).

Discussion

The most common and effective strategy to enhance the metabolic flux of a pathway towards the synthesis of a target metabolite(s) is to overcome the rate-limiting steps at the gene/enzyme level. This strategy may either aim to overexpress a limiting enzymatic step or downregulate the branch point diversion of a common intermediate or activate pathway-specific transcription factors/transporter proteins (Zhao and Verpoorte 2007; Guirimand et al. 2009). Nevertheless, our limited understanding of the regulatory architectures of the secondary metabolite biosynthetic pathways of plant and their biochemical integration with other metabolic networks generally makes it very difficult to envisage the outcomes of gene manipulation attempt carried out around a given biogenetic step. The introduction of one deliberate genetic alteration in a pathway may create many more limitations in the same or other overlapping routes (Qu et al. 2015; Liu et al. 2017). Since complete pathway gene expressions in medicinal crops are closely linked with tissue differentiation (viz., biosynthesis of dimeric TIAs vinblastine and vincristine remained highly restricted to the leaves of *C. roseus* that are the actual site for complete TIA pathway processing), it is always desirable to assess the overall influence of a genetic alteration at the whole plant level. The direct plant regeneration protocol optimized in this study assumes relevance in this context. De novo morphogenesis and regeneration of plants from cultured cells/tissues have always remained a critical impediment in transgenic research in *C. roseus* (Zhao and Verpoorte 2007; El-Sayed and Verpoorte 2007; Pan et al. 2012; Wang et al. 2012; Lu et al. 2016; Pan et al. 2016). This major limitation of *C. roseus* TIA pathway metabolic engineering approach is generally been ascribed to the genetically non-tractable and recalcitrant nature of in vitro plant regeneration via adventitious routes (Facchini and De Luca 2008; Verma et al. 2012). Therefore, the major milestone studies done for the genetic manipulation of TIAs in *C. roseus* were mainly carried out using the cell suspension, callus, and hairy root cultures, and due to a lesser degree of cellular organization, these cultures can only synthesize monomeric TIAs and failed to produce dimeric vinblastine and vincristine (Canel et al. 1998; Hughes et al. 2004a, b; Magnotta et al. 2007; Peebles et al. 2011; Liu et al. 2011; Kumar et al. 2011; van Moerkercke et al. 2015). Only a few efforts were made to bridge this technological gap of genetic engineering at whole plant level for TIA pathway modulation in *C. roseus* (Verma and Mathur 2011b; Pan et al. 2012; Wang et al. 2012). The direct plant regeneration protocol used in the present study was successfully utilized to generate and evaluate the whole plant level transgenics of *C. roseus* wherein two TIA pathway genes *CrTDC* and *CrSTR* were overexpressed. However, the present study also suffered from serious problems like leave browning followed by the death of leaves on regeneration medium due to high alkaloid content present inside the leaf explants and leaf death due to injuries caused by sonication. The pre-plasmolytic treatment of leaf explant in mannitol solution could have supported the cell survival and regeneration response by minimizing the concentration of antimitotic alkaloids (via ex-osmosis) in and around the dividing cells involved in the process of de novo shoot bud regeneration (Verma and Mathur 2011a, b). Alternately, this treatment can also influence the organogenesis by easy/rapid uptake of growth hormones by the explants through an osmotic pull.

The characterization data of four *CrTDC* and *CrSTR* engineered transgenic plants obtained in the present study offered some very interesting contrasts when compared with previous efforts carried out with cell suspensions, callus, and hairy root cultures. All transgenic plants showed less tryptophan content when compared to control shoots. This controlled accumulation of tryptophan in transgenic plants reflects the high *CrTDC* activity in them that was also confirmed by the presence of higher tryptamine content in all transgenic lines, compared to non-transgenic control. The secologanin estimation was also found marginally equal in all transgenic lines compared to control suggesting a balancing role in strictosidine production. These results from our study also suggested that improved TIA content in transgenic plants was mainly due to the higher availability of tryptamine and its rapid conversion towards secondary metabolism for TIA biosynthesis. A 65-fold and 38-fold higher *CrSTR* and *CrTDC* gene expression in the transformed plants efficiently modulates the flux of largely produced tryptamine towards the strictosidine biosynthesis and consequentially improved the monomeric catharanthine and vindoline and dimeric vinblastine production. The findings of the present study are in agreement with our earlier observation in *C. roseus* wherein higher monomeric and dimeric TIA accumulation in transformed

tissue was mainly due to the higher tryptamine availability and its rapid conversion to form different TIAs (Sharma et al. 2017b). It may be recalled here again that transgenic tissues of *C. roseus* overexpressing *TDC* and/or *STR* in earlier studies on callus or cell suspension could not result in similar improvement in TIA productivity as observed in the present study (Islas et al. 1994; Canel et al. 1998; Whitmer et al. 1998). Canel et al. (1998) observed a tenfold increase in *STR* and *TDC* activity in their undifferentiated transgenic cells with a detrimental effect on cell viability and growth. No increment in TIA content was noticed in these cells. A similar observation was also made by Goddijn et al. (1995) wherein the over expression of *TDC* alone in transgenic cell lines of *C. roseus* resulted in enhanced tryptamine content that failed to push the pathway flux towards TIA synthesis. Using a set of transgenic cell lines transformed with *TDC* and *STR* constructs, either alone or together (*STR*+*TDC*), Whitmer et al. (1998, 2002, 2003, 2004) conclusively suggested that TIA biosynthesis in *C. roseus* is more dependent on rapid turnover and utilization of tryptamine via its coupling with secologanin. This, in turn, requires the activation of iridoid flux towards the coupling steps. A study was done by Hughes et al. (2004a, b) with *crTDC* overexpressing hairy root cultures of *C. roseus* further reiterated this scenario. These workers also observed that when *crTDC* overexpressed alone in transformed roots, it showed no increment in tryptamine pool; however, when overexpressed along with ASα (anthranilate synthase) gene from Arabidopsis, it accumulated sixfold more tryptamine. Interestingly, more serpentine accumulation was observed in *crTDC*-alone overexpressed lines in comparison to no increment in *crTDC* + *ASα* expressing lines.

In our present investigation, the insertion of extra copies of *CrTDC* and *CrSTR* genes also demonstrated the enhanced vindoline and catharanthine production that is highly required for dimeric alkaloid vinblastine biosynthesis. Working with nine cell suspension lines, Saiman et al. (2014) have identified an important limiting step associated with the diversion of terpenoid pathway flux towards secologanin synthesis in *C. roseus* and reported that the cell line (CRPP-S) with higher TIA productivity recorded the higher frequency of *SGD* transcript in it. Sharma et al. (2017b) also achieved the higher levels of *CrSGD* gene expression in transiently transformed callus and leaf tissue overexpressing the same *CrTDC* and *CrSTR* gene construct and recorded up to twofold and up to fivefold enhanced *CrSGD* expression levels, respectively. Here, in our present investigation, transgenic plants expressing *CrTDC* and *CrSTR* genes recorded up to 21-fold enhanced *CrSGD* expression levels. Taking all the above-mentioned findings into considerations, it can be conclusively inferred that in *C. roseus*, (i) the presence of a larger tryptophan pool and its rapid conversion into tryptamine is crucial for initiating the TIA pathway; (ii) tryptamine utilization via its coupling with secologanin to synthesis strictosidine is a more limiting factor than their individual synthesis per se; and (iii) overexpression of *TDC*, *STR*, and *SGD* genes help to produce more strictosidine which is further channelized towards monomeric and dimeric TIA production in presence of a higher activity of strictosidine-β-D-glucosidase (SGD) enzyme.

The enhanced vindoline accumulation in transgenic *C. roseus* plants was also characterized by the increased activity of the *CrT30*, *T3R*, *D4H*, and *CrDAT* genes. The abundance of these gene transcripts in transgenic plants of *C. roseus* recovered in the present study was corroborated with high accumulation of vindoline in plant leaves. This was also in line with several earlier reports (De Luca et al. 1986, 2014; Wang et al. 2012; Thamm et al. 2016; Edge et al. 2017) A study conducted on *C. roseus* mutants demonstrated the importance of *T3O* and *T3R* along with the *DAT* and *D4H* gene activities in the formation of the vindoline from tabersonine in the leaves of *Catharanthus* plant (Edge et al. 2017). This effect of *STR*+*TDC* overexpression in our transgenic population was also shown in transgenic callus and intact leaf explants (Sharma et al. 2017b). On the other hand, lack of knowledge of steps leading to catharanthine biosynthesis impede the efforts of terpenoid indole alkaloid biosynthesis pathway mapping when the accumulation of catharanthine requires the correlation with genes of TIA pathway (Verma et al. 2012; Liu et al. 2017). The coupling of monomeric catharanthine and vindoline is required to synthesize the 3′,4′-anhydrovinblastine followed by vinblastine biosynthesis with the help of enhanced *CrPRX1* activity (Wang et al. 2012). In our present study, the transgenic lines AS3 and AS2 recorded the maximum vinblastine accumulation and exhibited up to sevenfold increased levels of *CrPRX1* transcripts. Recent studies have also shown that the active expression of downstream TIA pathway genes like *CrDAT* and *CrPRX1* is highly desired for the enhanced vinblastine biosynthesis in *C. roseus* (Wang et al. 2016; Sharma et al. 2017b). These results provide evidence that introduction of extra copies of upstream pathway genes like *TDC* and *STR* may not only result in increased TIA pathway activity but can also push the pathway towards vindoline, and to some extent up to the synthesis of dimeric TIAs.

Conclusion

The present study constitutes the first report of detailed molecular and biochemical characterization of leaf-derived whole plant transgenic of *C. roseus*. This genetic engineering effort to modulate TIA pathway demonstrated the high capabilities and efficacy of *CrTDC* and *CrSTR* to gain the enhanced antineoplastic alkaloid vinblastine synthesis in *C. roseus*. The direct plant regeneration method coupled with genetic transformation method showed high potential for studying the

molecular and biochemical basis of antineoplastic drug molecule vinblastine at whole plant level. Using the genetic transformation approach described here can be used to overexpress the different upstream and/or downstream TIA pathway genes in *C. roseus* to understand their expression pattern on TIA pathway biosynthesis. It has genetic engineering approach that has great implication because the expression of many TIA pathway genes is tightly associated with the higher level of cellular, tissue, and organ differentiation of a whole plant organization in *C. roseus*.

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Author's contribution AKM and AS conceived the idea and planned the work. AS conducted the experimental work. AS and PV analyzed the results and prepared the manuscript. AKM and AM read and edited the manuscript.

References

Canel C, Lopes-Cardoso MI, Whitmer S, Van der Fits L, Pasquali G, Van der Heijden R, Hoge JHC, Verpoorte R (1998) Effects of overexpression of strictosidine synthase and tryptophan decarboxylase on alkaloid production by cell cultures of *Catharanthus roseus*. Planta 205:414–419

Costa MMR, Hilliou F, Duarte P, Pereira LG, Almeida I, Leech M, Memelink J, Barcelo AR, Sottomayor M (2008) Molecular cloning and characterization of a vacuolar class III peroxidase involved in the metabolism of anticancer alkaloids in *Catharanthus roseus*. Plant Physiol 146:403–417

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

De Luca V, Salim V, Thamm A, Masada SA, Yu F (2014) Making iridoids/secoiridoids and monoterpenoid indole alkaloids: progress on pathway elucidation. Curr Opin Plant Biol 19:35–42

De Luca V, Balsevich J, Tyler RT, Eilert U, Panchuk BD, Kurz WGW (1986) Biosynthesis of indole alkaloids: developmental regulation of the biosynthetic pathway from tabersonine to vindoline in *Catharanthus roseus*. J Plant Physiol 125:147–156

Edge A, Qu A, Easson MLAE, Thamm AMK, Kim KH, De Luca V (2017) A tabersonine 3-reductase *Catharanthus roseus* mutant accumulates vindoline pathway intermediates. Planta 247:155–169. https://doi.org/10.1007/s00425-017-2775-8

El-Sayed M, Verpoorte R (2007) Catharanthus terpenoid indole alkaloids: biosynthesis and regulation. Phytochem Rev 6:277–305

Facchini PJ, De Luca V (2008) Opium poppy and Madagascar periwinkle: model non-model systems to investigate alkaloid biosynthesis in plants. Plant J 54:763–784

Facchini PJ, St-Pierre B (2005) Synthesis and trafficking of biosynthetic enzymes. Curr Opin Plant Biol 8:657–666

Geerlings A, Ibañez MM, Memelink J, van der Heijden R, Verpoorte R (2000) Molecular cloning and analysis of strictosidine beta-D-glucosidase, an enzyme in terpenoid indole alkaloid biosynthesis in *Catharanthus roseus*. J Biol Chem 275:3051–3056

Goddijn OJM, Pennings EJM, van der Helm P, Verpoorte R, Hoge JHC (1995) Overexpression of a tryptophan decarboxylase cDNA in *Catharanthus roseus* crown gall calluses results in increased tryptamine levels but not in increased terpenoid indole alkaloid production. Transgenic Res 4:315–323

Guirimand G, Burlat V, Oudin A, Lanoue A, St-Pierre B, Courdavault V (2009) Optimization of the transient transformation of *Catharanthus roseus* cells by particle bombardment and its application to the subcellular localization of hydroxymethyl butenyl 4-diphosphate synthase and geraniol 10-hydroxylase. Plant Cell Rep 28:1215–1234

Guirimand G, Courdavault V, Lanoue A, Mahroug S, Guihur A, Blanc N, Giglioli-Guivarc'h N, St-Pierre B, Burlat V (2010) Strictosidine activation in Apocynaceae: towards a "nuclear time bomb"? BMC Plant Biol 10:182–201

Guirimand G, Guihur A, Ginis O, Poutrain P, Héricourt F, Oudin A, Lanoue A, St-Pierre B, Burlat V, Courdavault V (2011a) The subcellular organization of strictosidine biosynthesis in *Catharanthus roseus* epidermis highlights several trans-tonoplast translocations of intermediate metabolites. FEBS J 278:749–763

Guirimand G, Guihur A, Poutrain P, Héricourt F, Mahroug S, St-Pierre B, Burlat V, Courdavault V (2011b) Spatial organization of the vindoline biosynthetic pathway in *Catharanthus roseus*. J Plant Physiol 168:549–557

Hughes RH, Shanks JV (2002) Metabolic engineering of plants for alkaloid production. Metab Eng 4:41–48

Hughes EH, Hong SB, Gibson SI, Shanks JV, San KY (2004a) Expression of a feedback-resistant anthranilate synthase in *Catharanthus roseus* hairy roots provides evidence for tight regulation of terpenoid indole alkaloid levels. Biotechnol Bioeng 86:718–727

Hughes EH, Hong SB, Gibson SI, Shanks JV, San KY (2004b) Metabolic engineering of the indole pathway in *Catharanthus roseus* hairy roots and increased accumulation of tryptamine and serpentine. Metab Eng 6:268–276

Islas I, Loyola-Vargas VM, Miranda-Ham ML (1994) Tryptophan decarboxylase activity in transformed roots from *Catharanthus roseus* and its relationship to tryptamine, ajmalicine, and catharanthine accumulation during the culture cycle. In Vitro Cell Dev Biol 30:81–83

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 13:3901–3907

Johanson DA (1940) Plant Microtechnique. Mc. Graw Hill Book. Co., New York, p 523

Kumar S, Bhatia S (2016) A polymorphic (GA/CT)n- SSR influences promoter activity of tryptophan decarboxylase gene in *Catharanthus roseus* L. Don. Sci Rep 6:33280

Kumar S, Jaggi M, Taneja J, Sinha AK (2011) Cloning and characterization of two new class III peroxidase genes from Catharanthus roseus. Plant Physiol Biochem 49:404–412

Liu DH, Ren WW, Cui LJ, Zhang LD, Sun XF, Tang KX (2011) Enhanced accumulation of catharanthine and vindoline in *Catharanthus roseus* hairy roots by overexpression of transcriptional factor ORCA2. Afr J Biotechnol 10:3260–3268

Liu J, Cai J, Wang R, Yang S (2017) Transcriptional regulation and transport of terpenoid indole alkaloid in *Catharanthus roseus*: exploration of new research directions. Int J Mol Sci 18:53

Lloyd G, McCown BH (1980) Commercially-feasible micropropagation of mountain laurel, *Kalmia atifolia*, by use of shoot-tip culture. Comb. Proc Int Plant Prop Soc Proc 30:421–427

Lu X, Tang K, Li P (2016) Plant metabolic engineering strategies for the production of pharmaceutical terpenoids. Front Plant Sci 7:1647. https://doi.org/10.3389/fpls.2016.01647

Magnotta M, Murata J, Chen J, De Luca V (2007) Expression of deacetylvindoline-4-O-acetyltransferase in *Catharanthus roseus* hairy roots. Phytochemistry 68:1922–1931

Mahroug S, Burlat V, St-Pierre B (2007) Cellular and subcellular organisation of the monoterpenoid indole alkaloid pathway in *Catharanthus roseus*. Phytochem Rev 6:363–381

Mathur AK, Mathur A, Seth R, Verma P, Vyas D (2006) Biotechnological interventions in designing specialty medicinal herbs for twenty-first century: some emerging trends in pathway modulation through metabolic engineering. In: Sharma RK, Arora R (eds) Herbal drugs: a twenty-first century perspective. Jaypee Brothers Medical Publishers, New Delhi, pp 83–94

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

Miettinen K, Dong L, Navrot N, Schneider T, Burlat V, Pollier J, Woittiez L, van der Krol S, Lugan R, Ilc T, Verpoorte R, Oksman-Caldentey KM, Martinoia E, Bouwmeester H, Goossens A, Memelink J, Werck-Reichhart D (2014) The seco-iridoid pathway from *Catharanthus roseus*. Nat Commun 5:3606. https://doi.org/10.1038/ncomms4606

Miralpeix B, Rischer H, Hakkinen TS, Ritala A, Seppanen-Laakso T, Kirsi-Marja OC, Capell T, Christou P (2013) Metabolic engineering of plant secondary products: which way forward ? Curr Pharma Des 19:5622–5639

Mishra S, Bansal S, Mishra B, Sangwan RS, Asha, Jadaun JS, Sangwan NS (2016) RNAi and homologous over-expression based functional approaches reveal triterpenoid synthase gene- cycloartenol synthase is involved in downstream withanolide biosynthesis in *Withania somnifera*. PLoS One 11(2):e0149691. https://doi.org/10.1371/journal.pone.0149691

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

Oudin A, Mahroug S, Courdavault V, Hervouet N, Zelwer C, Rodriguez-Conception M, St-Pierre B, Burlat V (2007) Spatial distribution and hormonal regulation of gene products from methyl erythritol phosphate and monoterpene secoiridoid pathways in *Catharanthus roseus*. Plant Mol Biol 65:13–30

Pan Q, Wang Q, Yuan F, Xing S, Zhao J, Choi YH, Verpoorte R, Tian Y, Wang G, Tang K (2012) Overexpression of ORCA3 and G10H in *Catharanthus roseus* plants regulated alkaloid biosynthesis and metabolism revealed by NMR-metabolomics. PLoS One 7:1–14

Pan Q, Rianika N, Mustafa Tang K, Choi YH, Verpoorte R (2016) Monoterpenoid indole alkaloids biosynthesis and its regulation in *Catharanthus roseus*: a literature review from genes to metabolites. Phytochem Rev 15:221–250

Pandey SS, Singh S, Babu CSV, Shanker K, Srivastava NK, Shukla AK, Kalra A (2016) Fungal endophyte of Catharanthus roseus enhance vindoline content by modulating structural and regulatory genes related to terpenoid indole alkaloids biosynthesis. Sci Rep 6:26583. https://doi.org/10.1038/srep26583

Pasquali G, Porto DD, Fett-Neto AG (2006) Metabolic engineering of cell cultures versus whole plant complexity in the production of bioactive monoterpene indole alkaloids: recent progress related to an old dilemma. J Biosci Bioeng 101:287–296

Peebles CAM, Sander GW, Hughes EH, Peacock R, Shanks JV, San KY (2011) The expression of 1-deoxy-D-xylulose synthase and geraniol-10-hydroxylase or anthranilate synthase increases terpenoid indole alkaloid accumulation in *Catharanthus roseus* hairy roots. Metab Eng 13:234–240

Qu Y, Michael LAEE, Jordan F, Razvan S, Tomas H, Luca VD (2015) Completion of the seven-step pathway from tabersonine to the anticancer drug precursor vindoline and its assembly in yeast. PNAS 112:6224–6229

Rischer H, Oresic M, Seppanen-Laakso T, Katajamaa M, Lammertyn F, Ardiles-Diaz W, Montagu MCE, Inzé D, Oksman-Caldentey KM, Goossens A (2006) Gene to metabolite networks for terpenoid indole alkaloid biosynthesis in *Catharanthus roseus* cells. PNAS USA 103:5614–5619

Saiman MZ, Mustafa NR, Pomahocova B, Verberne M, Verpoorte R, Choi YH, Schulte AE (2014) Analysis of metabolites in the terpenoid pathway of *Catharanthus roseus* cell suspensions. Plant Cell Tissue Organ Cult 117:225–239

Sharma A, Verma N, Verma P, Verma RK, Mathur A, Mathur AK (2017a) Optimization of a *Bacopa monnieri*-based genetic transformation model for testing the expression efficiency of pathway gene constructs of medicinal crops. In Vitro Cell Dev Bio Plants 53:22–32

Sharma A, Verma P, Mathur A, Mathur AK (2017b) Genetic engineering approach using early *Vinca* alkaloid biosynthesis genes led to increased tryptamine and terpenoid indole alkaloids biosynthesis in differentiating cultures of *Catharanthus roseus*. Protoplasma 255:425–435. https://doi.org/10.1007/s00709-017-1151-7

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

Trick HN, Finer JJ (1997) SAAT: sonication assisted *Agrobacterium*-mediated transformation. Transgenic Res 6:329–336

Thamm AMK, Qu Y, De Luca V (2016) Discovery and metabolic engineering of iridoid/secoiridoid and monoterpenoid indole alkaloid biosynthesis. Phytochem Rev 15(3):339–361

Twyman RM, Stoger E, Schillberg S, Christou P, Fischer R (2003) Molecular farming in plants: host systems and expression technology. Trends Biotechnol 2:570–578

Tyo KE, Alper HS, Stephanopoulos GN (2007) Expanding the metabolic engineering toolbox: more options to engineer cells. Trends Biotechnol 25:132–147

van der Heijden JDI, Snoeijer W, Hallard D, Verpoorte R (2004) The *Catharanthus* alkaloids: pharmacognosy and biotechnology. Curr Med Chem 11:607–628

van Moerkercke A, Steensma P, Schweizer F, Pollier J, Gariboldi I, Payne R, Bossche RV, Miettinen K, Espoz J, Purnama PC et al (2015) The bhlh transcription factor bis1 controls the iridoid branch of the monoterpenoid indole alkaloid pathway in *Catharanthus roseus*. PNAS USA 112:8130–8135

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

Verma P, Mathur AK (2011a) Direct shoot bud organogenesis and plant regeneration from leaf explants in *Catharanthus roseus*. Plant Cell Tissue Organ Cult 106:401–408

Verma P, Mathur AK (2011b) *Agrobacterium tumefaciens* mediated transgenic plant production via direct shoot bud organogenesis from pre-plasmolyzed leaf explants of *Catharanthus roseus*. Biotechnol Lett 33:1053–1060

Verma P, Mathur AK, Srivastava A, Mathur A (2012) Emerging trends in research on spatial and temporal organization of terpenoid indole alkaloid pathway in *Catharanthus roseus*: a literature update. Protoplasma 249:255–268

Verma P, Sharma A, Khan SA, Shanker K, Mathur AK (2015a) Overexpression of *Catharanthus roseus* tryptophan decarboxylase and strictosidine synthase in rol gene integrated transgenic cell suspensions of Vinca minor. Protoplasma 252:373–381

Verma P, Mathur AK, Khan SA, Verma N, Sharma A (2015b) Transgenic studies for modulating terpenoid indole alkaloids pathway in *Catharanthus roseus:* present status and future options. Phytochem Rev 16:19–54. https://doi.org/10.1007/s11101-015-9447-8

Verma P, Sharma A, Khan SA, Mathur AK, Shanker K (2014) Morphogenetic and chemical stability of long-term maintained -mediated transgenic plants. Nat Prod Res 29(4):315–320

Verpoorte R, Alfermann AW (2000) In: Verpoorte R, Alfermann AW (eds) Metabolic engineering of plant secondary metabolism. Kluwer Academic Publishers, Dordrecht

Verpoorte R, van der Heijden R, Moreno PRH (1997) Biosynthesis of terpenoid indole alkaloids in *Catharanthus roseus*cells. In: Cordell GA (ed) The alkaloids, vol 49. Academic, San Diego, pp 221–299

Wang Q, Xing S, Pan Q, Yuan F, Zhao J, Tian Y, Chen Y, Wang G, Tang K (2012) Development of efficient *Catharanthus roseus* regeneration and transformation system using *Agrobacterium tumefaciens* and hypocotyls as explants. BMC Biotechol 12:34–46

Wang X, Pan YJ, Chang BW, Hu YB, Guo XR, Tang ZH (2016) Ethylene-induced vinblastine accumulation is related to activated expression of downstream TIA pathway genes in *Catharanthus roseus*. Bio Med Res Int 2016:1–8. https://doi.org/10.1155/2016/3708187

Whitmer S, Canel C, Hallard D, Goncalves C, Verpoorte R (1998) Influence of precursor availability on alkaloid accumulation by transgenic cell line of *Catharanthus roseus*. Plant Physiol 116: 853–857

Whitmer S, van der Heijden R, Verpoorte R (2002) Effect of precursor feeding on alkaloid accumulation by a strictosidine synthase over-expressing transgenic cell line S1 of *Catharanthus roseus*. Plant Cell Tissue Organ Cult 69:85–93

Whitmer S, van der Heijden R, Verpoorte R (2003) Effect of precursor feeding on alkaloid accumulation by a tryptophan decarboxylase overexpressing transgenic cell line T22 of *Catharanthus roseus*. J Biotechnol 96:193–203

Whitmer S, Canel C, van der Heijden R, Verpoorte R (2004) Long-term instability of alkaloid production by stably transformed cell lines of *Catharanthus roseus*. Plant Cell Tissue Organ Cult 74:73–80

Zhao J, Verpoorte R (2007) Manipulating indole alkaloid production by *Catharanthus roseus* cell cultures in bioreactors: from biochemical processing to metabolic engineering. Phytochem Rev 6:435–457