Plant Science 239 (2015) 56–66

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Precursor feeding studies and molecular characterization of *geraniol synthase* establish the limiting role of geraniol in monoterpene indole alkaloid biosynthesis in *Catharanthus roseus* leaves

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ARTICLE INFO

Article history:
Received 1 February 2015
Received in revised form 1 July 2015
Accepted 10 July 2015
Available online 19 July 2015

Keywords:
Catharanthus roseus
Geraniol
Gene expression
Geraniol synthase
Monoterpene indole alkaloids
Virus-induced gene silencing

ABSTRACT

The monoterpene indole alkaloids (MIAs) are generally derived from strictosidine, which is formed by condensation of the terpene moiety secologanin and the indole moiety tryptamine. There are conflicting reports on the limitation of either terpene or indole moiety in the production of MIAs in *Catharanthus roseus* cell cultures. Formation of geraniol by geraniol synthase (GES) is the first step in secologanin biosynthesis. In this study, feeding of *C. roseus* leaves with geraniol, but not tryptophan (precursor for tryptamine), increased the accumulation of the MIAs catharanthine and vindoline, indicating the limitation of geraniol in MIA biosynthesis. This was further validated by molecular and *in planta* characterization of *C. roseus GES* (*CrGES*). *CrGES* transcripts exhibited leaf and shoot specific expression and were induced by methyl jasmonate. Virus-induced gene silencing (VIGS) of *CrGES* significantly reduced the MIA content, which was restored to near-WT levels upon geraniol feeding. Moreover, over-expression of *CrGES* in *C. roseus* leaves increased MIA content. Further, *CrGES* exhibited correlation with MIA levels in leaves of different *C. roseus* cultivars and has significantly lower expression relative to other pathway genes. These results demonstrated that the transcriptional regulation of *CrGES* and thus, the *in planta* geraniol availability plays crucial role in MIA biosynthesis.

1. Introduction

Madagascar periwinkle (*Catharanthus roseus*, family Apocynaceae) is an important tropical medicinal plant, which accumulates an array of diverse compounds comprising over 130 different MIAs [1]. *C. roseus* has the unique distinction of producing two pharmacologically important antineoplastic dimeric MIAs, vinblastine and vincristine, which are extensively used for treatment of various types of cancers [1]. Both dimeric MIAs are produced in a leaf-specific manner through coupling of monomeric MIAs (vindoline and catharanthine), and are present in extremely low concentration (0.0002% FW) [2]. *C. roseus* also accumulates other important MIAs such as ajmalicine and serpentine, which are used for the treatment of hypertension and cardiovascular diseases [3].

The MIA biosynthesis in *C. roseus* is highly complex with more than 50 biosynthetic events comprising pathway enzymes, regulators, and intra-/intercellular signalling coupled with transport of metabolites [4]. It involves several sub-pathways, which includes methylerythritol phosphate (MEP), indole, secoiridoid (terpene), and finally the MIA pathway itself (Fig. 1). Strictosidine formed by condensation of indole pathway-derived tryptamine and terpene/secoiridoid pathway-derived secologanin, acts as the most common MIA precursor in plants [5–7] (Fig. 1). The formation of secologanin starts with the conversion of the monoterpene geraniol by geraniol 10-hydroxylase/8-oxidase (G8O) to 8-hydroxygeraniol, which is ultimately converted to secologanin *via* multiple enzymatic steps [2]. Plants produce monoterpenes *via* the plastidial MEP pathway, which provides isopentenyl diphosphate (IPP) and its isomer dimethylally diphosphate (DMAPP) substrates for geranyl diphosphate (GPP) formation by GPP synthase [8,9]. The GPP thus,

Abbreviations: EV, empty vector; G8O, geraniol 10-hydroxylase/8-oxidase; GES, *geraniol synthase*; GGPP, geranylgeranyl diphosphate; GPP, geranyl diphosphate; MeJA, methyl jasmonate; MEP, methylerythritol phosphate; MIA, monoterpene indole alkaloids; MPGR, medicinal plant genomics resource database; ORCA, octadecanoid-responsive *Catharanthus* AP2/ERF domain; qRT-PCR, quantitative real-time PCR; VIGS, virus-induced gene silencing; YFP, yellow fluorescent protein.

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http://dx.doi.org/10.1016/j.plantsci.2015.07.007
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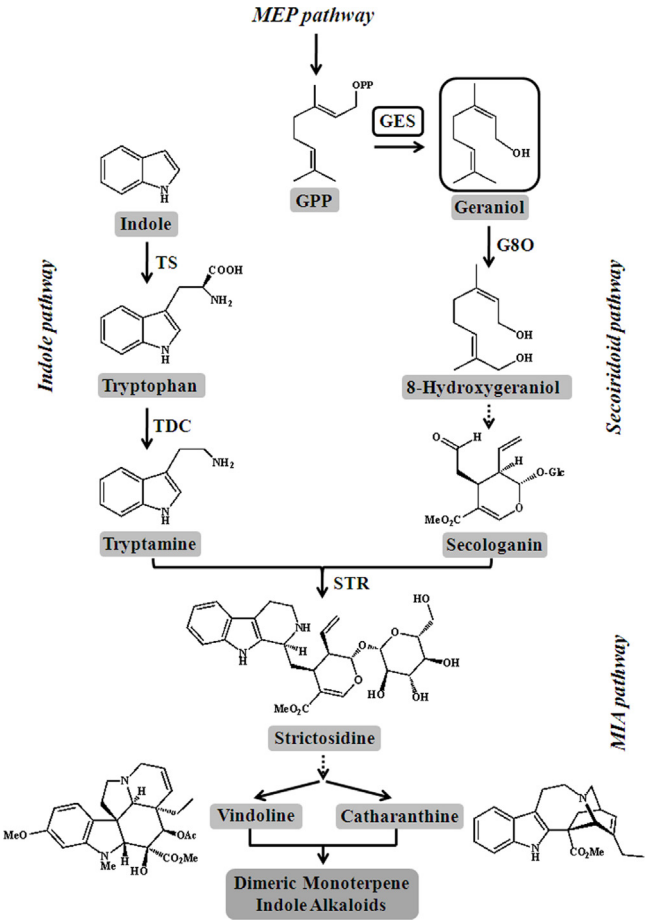

Fig. 1. Simplified view of MIA biosynthesis in *C. roseus*.
Full and dashed arrows indicate single and multiple enzymatic steps, respectively. The monoterpene branching step, GES and its product geraniol are boxed. The name of enzyme involved in each step is indicated on the right side of arrow. The intermediates and endproducts are shown in grey background. GES, geraniol synthase; G8O, geraniol-10-hydroxylase/8-oxidase; GPP, geranyl diphosphate; SLS, secologanin synthase; STR, strictosidine synthase; TDC, tryptophan decarboxylase; TS, tryptophan synthase. Names of pathways are in bold italics.

formed is utilized either by geranylgeranyl diphosphate (GGPP) synthase that provides precursor for diterpenes and tetraterpenes of primary/secondary metabolism or is channeled into monoterpene biosynthesis by monoterpene synthases, which based on their enzyme function, convert GPP into diverse terpene skeletons. In plants, geraniol formation from GPP was initially thought to be carried out by the action of either a phosphatase- or monoterpene synthase-based catalysis. Isolation of the gene encoding GES from sweet basil (*Ocimum basilicum*) provided the first evidence of the involvement of a monoterpene synthase in geraniol formation as a component of essential oil [10]. Subsequently, few more GESs have been isolated and functionally characterized from *Cinnamomum tenuipilum*, *Valeriana officinalis*, *Lippia dulcis*, *Perilla* sp., all of which accumulate geraniol in their essential oils [11–13]. Recently a gene encoding GES was functionally characterised in *C. roseus* [14]. The GES-YFP fusion studies showed that it was localized in plastids and the recombinant GES produced in *Escherichia coli* catalyzed the *in vitro* conversion of GPP into geraniol [14].

Although synthetic biology approaches for producing MIA pathway intermediates such as strictosidine and vindoline have been reported recently in yeast [15,16], metabolic engineering efforts at the whole plant level have yielded little success. Previous reports on precursor feeding of indole or terpenoid building blocks in cell and hairy root cultures of *C. roseus* have shown largely inconsistent results underscoring the difficulties associated with the study of complex MIA pathway [17–22]. It was suggested that the effect of precursor feeding depends on the metabolic status of cell lines that influences the steady-state concentration of a particular metabolite [21,23]. Despite the fact that leaves are the major sites of monomeric vindoline and dimeric vinblastine and vincristine, studies on the availability of terpene or indole precursors in this tissue have not been carried out. Although GES has been isolated and characterized *in vitro*, genetic proof for its involvement in MIA biosynthesis is lacking in *C. roseus*. Hence, this study was taken up to explore the limitation of indole/terpene (tryptophan/geraniol) moiety on leaf MIA content. Increased accumulation of MIAs in geraniol fed leaves led us to characterize the *in planta* role of GES, the enzyme responsible for the formation of geraniol, in MIA biosynthesis in *C. roseus* leaves. Spatio-temporal transcript distribution of *CrGES*, its virus-induced gene silencing (VIGS) as well as transient over-expression, biochemical complementation, and comparative gene expression analysis, provided the *in planta* evidence for the critical role of geraniol in MIA biosynthesis.

2. Materials and methods

2.1. Plant material

C. roseus cv. Nirmal and cv. Dhawal (National Gene Bank for Medicinal and Aromatic Plants at CSIR-CIMAP, Lucknow, India) plants were grown under normal greenhouse conditions. For VIGS/transient over-expression experiments *C. roseus* cv Dhawal seeds were germinated and grown either in a greenhouse or a growth room with 16/8-h light/dark photoperiod at 25 °C for 3–6 weeks until the plants had at least two true leaf pairs.

2.2. MeJA treatment and precursor feeding

For MeJA treatment, 95% pure MeJA (Sigma–Aldrich, USA) was dissolved in dimethyl sulphoxide (0.2% DMSO) to a final concentration of 200 μM. Excised leaves were dipped in MeJA or 0.2% DMSO (control) solutions and placed in 5% sucrose solution. Samples were collected at 0, 1, 4, 8, and 12 h, and stored at −80 °C until further use. For geraniol and nerol feeding, stock solutions of 56.9 mM and 57.0 mM, respectively, were prepared using authentic standards (Sigma–Aldrich, USA) in DMSO and the stock was added to deionised water to achieve different dilutions. DMSO solution (350 μl in 10 ml deionised water) was used as control. For tryptophan feeding, 5 mM tryptophan (Himedia Laboratories, India) stock was prepared in deionised water. For feeding experiments, first fully developed leaf pairs were infiltrated with different concentrations (0.0, 0.1, 0.5, 1.0 and 2.0 mM) of either geraniol or tryptophan using needleless syringe. Post-infiltration, leaves were covered with Klin film to maintain the humidity. After 48 h, leaves were harvested and dried for alkaloid extraction.

2.3. Gene cloning and construction of VIGS vectors

The pTRV1 and pTRV2 VIGS vectors [24] were procured from The Arabidopsis Information Resource (TAIR), USA. The 500 bp fragments of *PDS* and *GES* of *C. roseus* were amplified by RT-PCR using cDNA prepared from leaf RNA with gene specific primers (Table S1). To facilitate cloning into pTRV vectors, both primers of *Phytoene desaturase* (*PDS*) contained the *Eco*RI site, whereas the forward and reverse primers of *GES* consisted *Xba*I and *Xho*I sites, respectively. The amplified fragments were cloned into pJET1.2/vector (Thermo Scientific INC, Canada) and sequences were confirmed by nucleotide sequencing using ABI 3130 genetic analyzer (Applied Biosystems, USA). Later, these fragments were restriction digested

and sub-cloned into pTRV2 vector resulting in pTRV-*CrPDS* and pTRV-*CrGES* constructs (Fig. S1)

2.4. Agrobacterium *infiltration*

Agrobacterium tumefaciens strain GV3101 was transformed with pTRV1, pTRV2 and pTRV derived constructs by freeze-thaw method [25]. The transformed *Agrobacteria* cultures were grown in 100 ml YEP medium at 28 °C. The overnight grown cultures were harvested by centrifugation and resuspended in infiltration buffer (10 mM $MgCl_2$, 10 mM MES, 200 μM acetosyringone, pH 5.6) to a final OD_{600} of 1.6 and incubated at 28 °C for 5–6 h. For leaf infiltration, mixture of *Agrobacterium* cultures containing TRV1 and TRV2 or its derivatives were mixed in 1:1 ratio and were infiltrated by pinching below apical meristem [26] of 4-6 leaf staged plants with a dissecting needle. Post-infiltration, plants were kept in growth room. First fully expanded leaves were collected after 21 days post infiltration (dpi) and stored at −80 °C for further analysis.

2.5. *Transient overexpression of* CrGES *in* C. roseus *leaves*

The coding region of *CrGES* was PCR-amplified using specific forward and reverse primers (Table S1). The amplified fragment was cloned into pJET1.2/vector and sequences were confirmed by nucleotide sequencing. Later the fragment was restriction digested and subcloned into the *Xba*I and *Sac*I sites by replacing the β-glucouronidase (GUS) gene of the pBI121 binary vector resulting in pBI121:*CrGES* construct (Fig. S1). Transient over-expression was performed with *A. tumefaciens* strain GV3101 harboring pBI121-*CrGES* and pBI121 vectors (control). Briefly, overnight *Agrobacteria* cultures were pelleted and resuspended in infiltration buffer (50 mM MES pH 5.6, 2 mM Na_3PO_4, 0.5% glucose and 100 μM acetosyringone) to a final OD_{600} of 0.15–0.2. The suspension was further incubated at 28 °C for 4 h prior to infiltration. *Agrobacteria* cultures were infiltrated into the first pair of leaves using a needleless syringe. To facilitate the infiltration, leaves were pinched with a needle on the underside. Plants were covered and maintained in the dark for 48 h. Leaves were harvested and stored at −80 °C until further analysis.

2.6. *RNA isolation and expression analysis*

Total RNA was extracted from 100 mg plant tissue using Spectrum™ Plant Total RNA Kit (Sigma–Aldrich, USA) according to the manufacturer's instructions. In all cases, on-column DNase digestion was performed to remove trace amounts of DNA using DNase I (Sigma–Aldrich, USA). The DNA-free total RNA was quantified by UV-spectrophotometer (Kinetic Biospectrometer, Eppendorf, Germany). Five microgram of total RNA was used for first-strand cDNA synthesis with oligo$(dT)_{18}$ primers using RevertAid H Minus Reverse Transcriptase (Thermo scientific Inc., Canada). The *RPS9* gene served as an endogenous control for expression studies. Semi quantitative RT-PCR was performed for *GES* and *RPS9* using standard PCR conditions. PCR products were separated on a 1% TAE gel and visualized by ethidium bromide staining. Real-time qRT-PCR was performed using a linear range of cDNA and specific primers for each gene (Table S1). qRT-PCR was performed in a 384-well plate using the Applied Biosystems 7900HT Fast Real-Time PCR System (PE Applied Biosystems, USA) with SYBR green fluorescent dye. qRT-PCR conditions were as follows: 94 °C for 10 min for one cycle, followed by 40 cycles of 94 °C for 15 s, 54 °C for 15 s and 72 °C for 15 s. Fold change differences in gene expression were analyzed using the comparative cycle threshold (Ct) method (Applied Biosystems, USA).

2.7. *Alkaloid analysis and quantification*

Alkaloids from *C. roseus* leaves were extracted following the protocol of Miranda-Ham et al. [27] and Suttipanta et al. [28]. About 5–10 mg dried leaf tissue was ground in methanol and incubated at 55 °C for 2 h with occasional shaking. The methanolic extract was filtered and evaporated to dryness. The residue was dissolved in 2.5% H_2SO_4 and was extracted twice with equal volume of ethyl acetate, retaining the aqueous phase each time. The pH of the aqueous extract was adjusted to 9.0 using NH_4OH. The alkaloids were extracted with equal volume of ethyl acetate and evaporated to dryness. The residue containing alkaloids was dissolved in methanol and was used for analysis by HPLC using Waters Prep LC system (Waters, Milford, MA, USA) equipped with 2996 photodiode array (PDA) detector and 600E pump. Alkaloid extract (20 μl) was injected onto a C_{18} symmetry reverse phase column (5 μm, 250 × 4.6 mm, Waters, Milford, MA, USA). The mobile phase consisted of mixture of 100 mM ammonium acetate ($NH_4C_2H_3O_2$) buffer (pH 7.3) and acetonitrile (C_2H_3N). Briefly, for the first 5 min flow rate of mobile phase (70:30 ratio of $NH_4C_2H_3O_2$: C_2H_3N) was maintained at 1 ml/min and then was linearly ramped to 36:64 for the next 5 min with flow rate of 1.4 ml/min. Subsequently, the ratio was changed to 20:80 with the same flow rate of 1.4 ml/min for next 5 min. Finally, the flow rate was reduced to 1 ml/min with 70:30 ratio for last 5 min. PDA data was extracted at 254 nm to quantify vindoline and catharanthine. Authentic standards were used for the identification and quantification of vindoline and catharanthine (Fig. S2a). Analysis was performed with Empower Pro software (Waters, Milford, MA, USA). Peak area obtained from authentic standards (Sigma–Aldrich, St. Louis, MO, USA) and samples were used to calculate the amount of alkaloids and expressed as mg/g dry weight or relative content in % of vindoline/catharanthine.

The alkaloid extracts were further analyzed using LC-ESI–MS/MS with a Thermo TSQ Quantum Access Max Triple Quadruple MS/MS device. LC was carried out for vinblastine on a C18 column (Merck PuroSPHER 2 μm, 100 × 2.1 mm) with a flow rate of 150 μl min^{-1} in Accela High Speed Pump equipped with Thermo Accella Auto sampler. The mobile phase consisted of different ratios of methanol: 0.1% formic acid. For the first 4 min the mobile phase ratio was set to 5:95, followed by 50:50 for next 2 min. Subsequently the ratio was changed to 90:10 till 9th min followed by 30:70 up to 11th min. Finally the mobile phase ratio was adjusted to 5:95 till the end (13th min). The column elution was coupled to MS analysis, which was carried out on Thermo Fisher TSQ Quantum Access MAX Spectrometer. The MS/MS detector equipped with an ESI system was operated with a capillary voltage of 2 to 6 kV and heated at 300 °C. Nitrogen was used as a flowing sheath gas at a pressure of 20 and as auxiliary gas at a pressure of 50. Analysis was performed in scan mode ranging from mass-to-charge ratio 100–900. Collision-induced dissociation experiments were performed in the ion trap using helium as the collision gas. The collision energy varied from 10–45 V. The isolation width of the parent ion for following MS fragmentation events was set at 0.5. Alkaloids were verified by their MS/MS spectra compared with authentic standards. Vinblastine was detected at RT of 7.9–8.0 min with following fragmentation pattern; +MS: 811.4 $[M+H]^+$, +MS2(811.4):336.950, 751.050 (Fig. S2b).

2.8. *Statistical analysis*

Mean, standard error and number of replicates were used for statistical evaluation using GraphPad QuickCalc online software (http://www.graphpad.com/quickcalcs/ttest1.cfm). The statistical significance of differences between control and treated samples was tested by unpaired Student's *t*-test.

3. Results

3.1. Feeding of geraniol, but not tryptophan increases accumulation of catharanthine and vindoline in C. roseus leaves

It has been reported that feeding of precursors including geraniol and tryptophan in hairy root cultures and suspension cultures of *C. roseus* affects alkaloid accumulation [21,22]. However, no such information exists in *C. roseus* leaves, which are the main source of monomeric vindoline, and dimeric vinblastine and vincristine. Hence, to determine whether starting precursors of terpenoid and indole moieties are limiting for MIA formation in *C. roseus* leaves, different concentrations of geraniol and tryptophan were infiltrated into the 1st pair of leaves on the plant. In the case of geraniol feeding, to rule out the non-specific effect of monoterpene feeding on MIA accumulation, nerol, an isomer of geraniol was used as negative control. Quantification of catharanthine and vindoline accumulation 48 h post-infiltration exhibited an increasing trend in their accumulation corresponding to geraniol concentration, whereas there was no drastic effect of nerol feeding on their levels (Figs. 2b and S3b). At 0.1 mM geraniol, there was an increase of ~1.7-fold in catharanthine and vindoline content, which reached its maximum at 0.5 mM (~2.2-fold) and slightly decreased thereafter at 1.0 (~1.9-fold) and 2.0 mM (~1.6-fold) geraniol concentration (Figs. 2a and S3a). However, unlike the positive effect of geraniol feeding on MIAs, tryptophan feeding exhibited little effect on MIA accumulation in *C. roseus* leaves (Figs. 2c and S3c).

3.2. Spatio-temporal expression of (CrGES)

The biosynthesis of MIAs in *C. roseus* is regulated in a tissue specific and developmental manner as well is induced by methyl jasmonate (MeJA, a known alkaloid pathway regulator). Hence, to determine the contribution of *CrGES* in MIA formation, its transcript abundance was analyzed in different tissues and in MeJA-treated leaves by quantitative real-time PCR (qRT-PCR) using gene-specific primers. The expression of *CrGES* was highest in the leaf followed by shoot, which are the parts of the plant that have previously been shown to accumulate key MIAs (Fig. 3a). *CrGES* also showed relatively significant expression in flower, whereas a negligible level of transcripts was found in silique and root (Fig. 3a). In order to determine whether *CrGES* expression in leaf is induced in response to MeJA, transcript levels were measured over a 12 h time course after leaves were treated with MeJA. Relative expression levels of *CrGES* were compared with those of control. *CrGES* expression exhibited a gradual increase over time reaching its maximum (~5-fold) at 8 h and showed a steady decline (~half level of induction at 8 h) at 12 h (Fig. 3b).

3.3. Silencing of CrGES reduced catharanthine, vindoline, and vinblastine accumulation

VIGS technique in *C. roseus* has been utilized to advance the understanding of the MIA metabolism in leaves [26,29]. Hence, to assess the involvement of *CrGES* in MIA biosynthesis, a virus-induced gene silencing (VIGS) approach was adapted in *C. roseus* leaves utilizing pTRV vector system [30]. A 500 bp amplicon corresponding to the coding region of *CrGES* was amplified from leaf cDNA using specific primers and cloned into pTRV2 vector resulting in pTRV2-*CrGES* construct (Fig. S1), which was used for silencing studies in *C. roseus* cv Dhawal. Parallel infiltration experiments with pTRV2-*CrPDS* construct was performed to silence the phytoene desaturase (*PDS*) gene that would result in photobleaching and serve as a visual marker to collect leaf tissue from *GES*-silenced plants for transcript and metabolite analysis (Fig. 4a). The degree of VIGS was determined at the transcript level by analyzing the

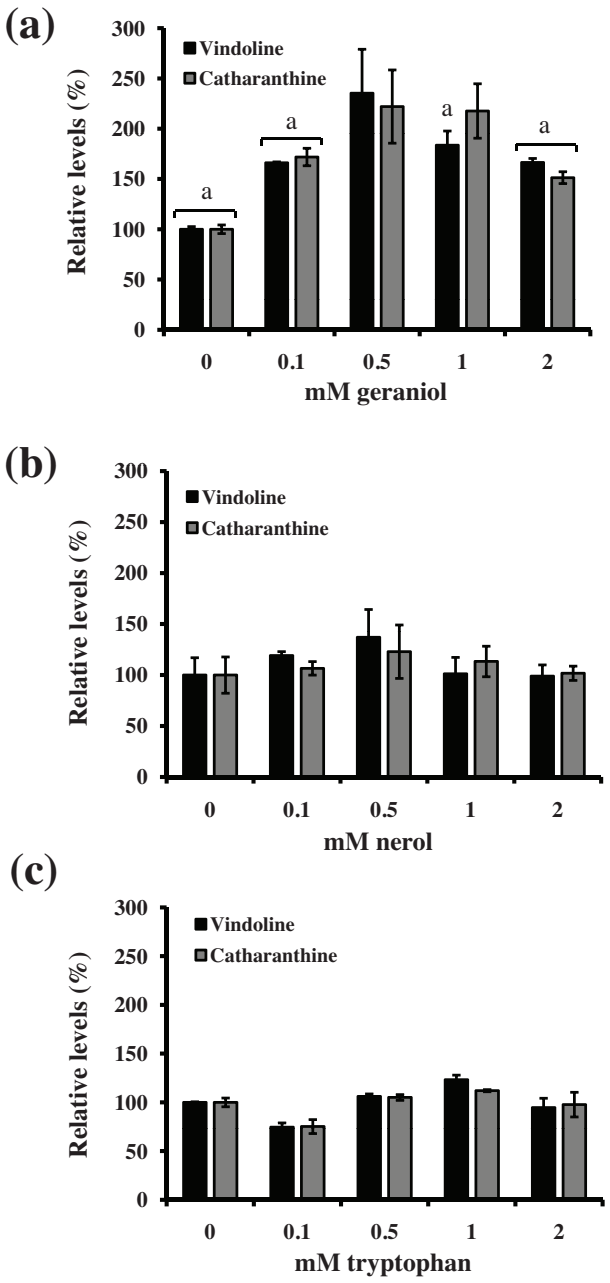

Fig. 2. Effect of geraniol, nerol and tryptophan feeding on MIA accumulation in *C. roseus*.
First leaf pairs of *C. roseus* were infiltrated with different concentrations (0.0, 0.1, 0.5, 1.0 and 2.0 mM) of geraniol (a), nerol (b), and tryptophan (c). Leaf samples were collected 48 h post-infiltration for alkaloid extraction. Extracted alkaloids were analyzed and quantified by HPLC and represented as % relative levels of vindoline (black bar) and catharanthine (grey bar). The data represents mean ± standard error (SE) values of two independent experiments with at least two technical replicates. "a" indicates significant difference between groups at $P < 0.05$.

CrGES mRNA accumulation by semi-quantitative RT-PCR using gene specific primers. Analysis of amplified samples indicated clear suppression of *CrGES* expression (Fig. 4b). In control TRV plants, RT-PCR product of *GES* gene was visible from 30 cycles whereas in *CrGES*-vigs plants the clear visibility of amplicons was delayed up to 36 cycles, indicating suppression of *CrGES* expression (Fig. 4b). Further, transcript analysis of *CrGES* monitored by quantitative real-time PCR (qRT-PCR) revealed that the transcript level was decreased by approximately 80% in *CrGES*-vigs plants as compared to those of empty vectors (EV) confirming efficient silencing of

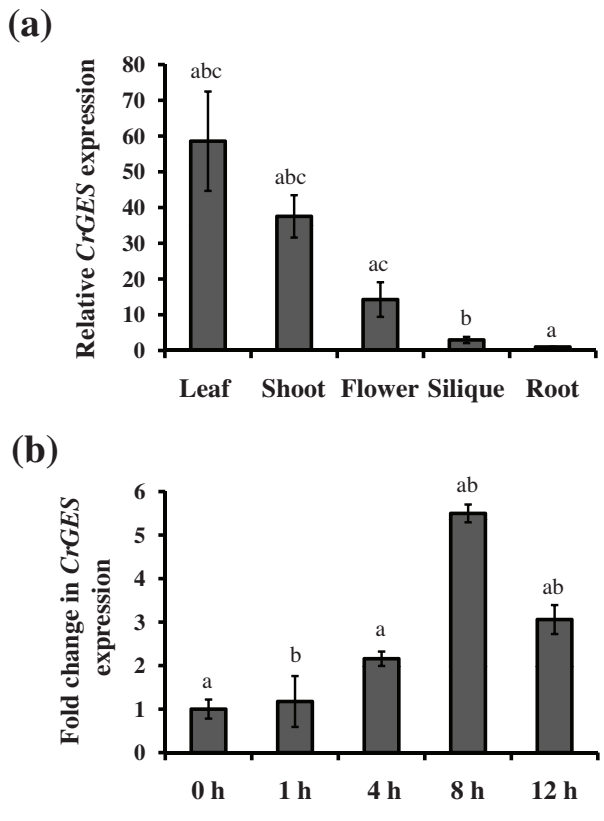

Fig. 3. Expression analysis of *CrGES* in different tissues of *C. roseus* and in response to MeJA treatment in leaves.
(a) qRT-PCR analysis of *CrGES* expression in different tissues of *C. roseus*. *CrGES* mRNA expression in roots (least expressing tissue) was set to 1 to determine the relative abundances of mRNA transcripts. (b) qRT-PCR analysis of *CrGES* gene expression in *C. roseus* leaves treated with 200 μM MeJA at 0, 1, 4, 8, and 12 h. Expression level is displayed as relative expression compared to untreated leaves. *CrRPS9* was used as endogenous control for normalization. In both experiments, each data point represents the mean ± standard error (SE) of two independent experiments with two technical replicates. Same letters indicate significant difference between groups at $P < 0.05$.

CrGES (Fig. 4c). There was no significant effect on the transcript levels of other MIA pathway genes like *G8O*, *STR*, *TDC* as well as *ORCA3* transcription factor in *CrGES*-vigs plants (Fig. 4c). Alkaloid analysis by HPLC in leaf tissues showed that the *CrGES* silencing was accompanied by reduction in monomeric and dimeric MIAs as compared to the empty-vector controls (Fig. 5). While catharanthine level was decreased by approximately 49%, vindoline accumulation was reduced by 57% in *CrGES*-vigs compared to EV control (Fig. 5b). Further analysis of vinblastine by LC–MS/MS analysis showed ~60% reduction in *CrGES*-vigs compared to the EV control (Figs. 5c and S2b).

3.4. *Feeding of* CrGES-*vigs leaves with geraniol restores catharanthine and vindoline levels*

Reduced accumulation of catharanthine, vindoline and vinblastine in *CrGES*-vigs tissues (Fig. 5) strongly suggested that there was reduced formation of geraniol and its availability as an endogenous starting precursor. Since geraniol feeding in leaves increased the MIA content (Figs. 2 and 5), we fed *CrGES*-vigs leaves with 0.5 mM geraniol to determine whether it would complement the silencing effect on MIA accumulation (Fig. 5). Analysis of alkaloids by HPLC showed an increased accumulation of both catharanthine and vindoline in geraniol-fed *CrGES*-vigs tissue compared to *CrGES*-vigs leaves without geraniol infiltration (Fig. 5). Further quantification revealed that geraniol feeding

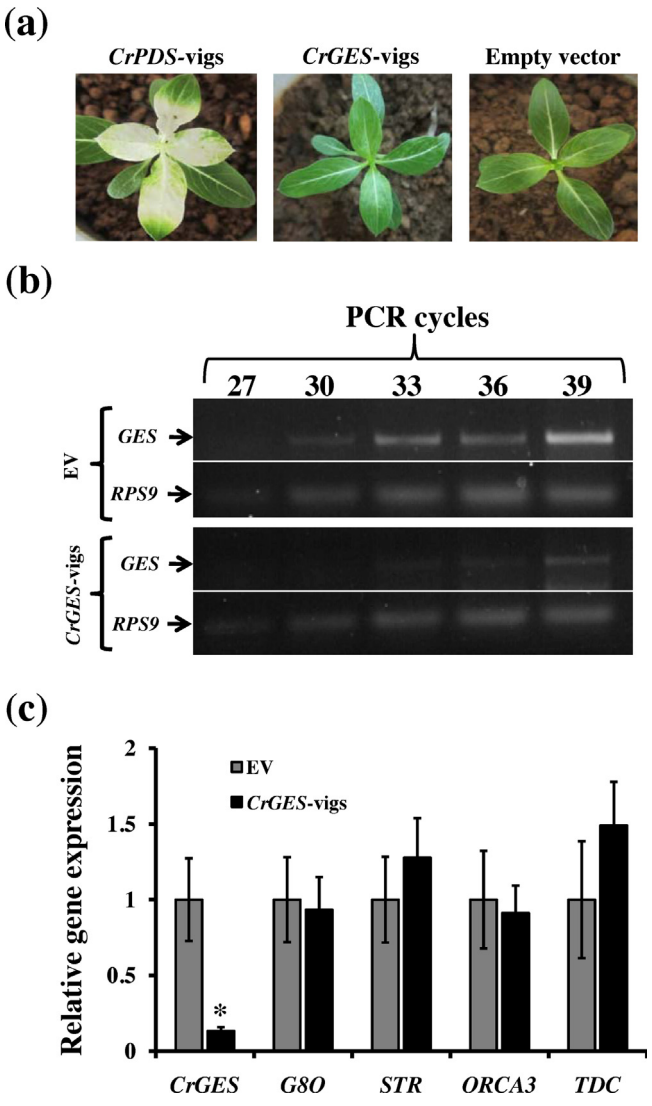

Fig. 4. Tobacco rattle virus (TRV)-mediated silencing of *CrGES* in *C. roseus*.
(a) Representative *C. roseus* plants 3 weeks post-infiltration with 1:1 of pTRV1/pTRV2-*CrPDS* (*CrPDS*-vigs), pTRV1/pTRV2-*CrGES* (*CrGES*-vigs) and pTRV1/pTRV2 (empty vector, EV). (b) Expression analysis of *CrGES* and *RPS9* by semi-quantitative RT-PCR using total RNA isolated from EV and *CrGES*-vigs plants. The level of amplicons from different PCR cycles (indicated on top of the lane) is shown. Arrows indicate amplicons of *CrGES* (750 bp) and *RPS9* (100 bp). (c) Expression of *CrGES*, *G8O*, *STR*, *ORCA3*, and *TDC* was determined by qRT-PCR analyses using total RNA extracted from *C. roseus* leaves of *CrGES*-vigs plants (black bars) relative to EV control (grey bars; normalized to 1). For all calculations *CrRPS9* was used as a reference gene. Data represents an average of two to three independent experiments with two technical replicates. Significant difference at $P < 0.05$ are indicated by '*'.

complemented the silencing effect on MIAs, which was comparable to the catharanthine and vindoline content in EV control leaves (Fig. 5b). The recovery of catharanthine and vindoline levels in geraniol-fed *CrGES*-vigs tissues further provided compelling proof that *CrGES*-vigs plants had reduced formation of geraniol (Fig. 5).

3.5. *Transient over-expression of* CrGES *in* C. roseus *leaves increases catharanthine and vindoline content*

In *C. roseus*, transient over-expression strategy has been utilized to validate gene function [31–33]. Since VIGS of *CrGES* in *C. roseus* leaves resulted in reduced MIA content (Figs. 4 and 5), we checked whether over-expression of *CrGES* in leaves can improve

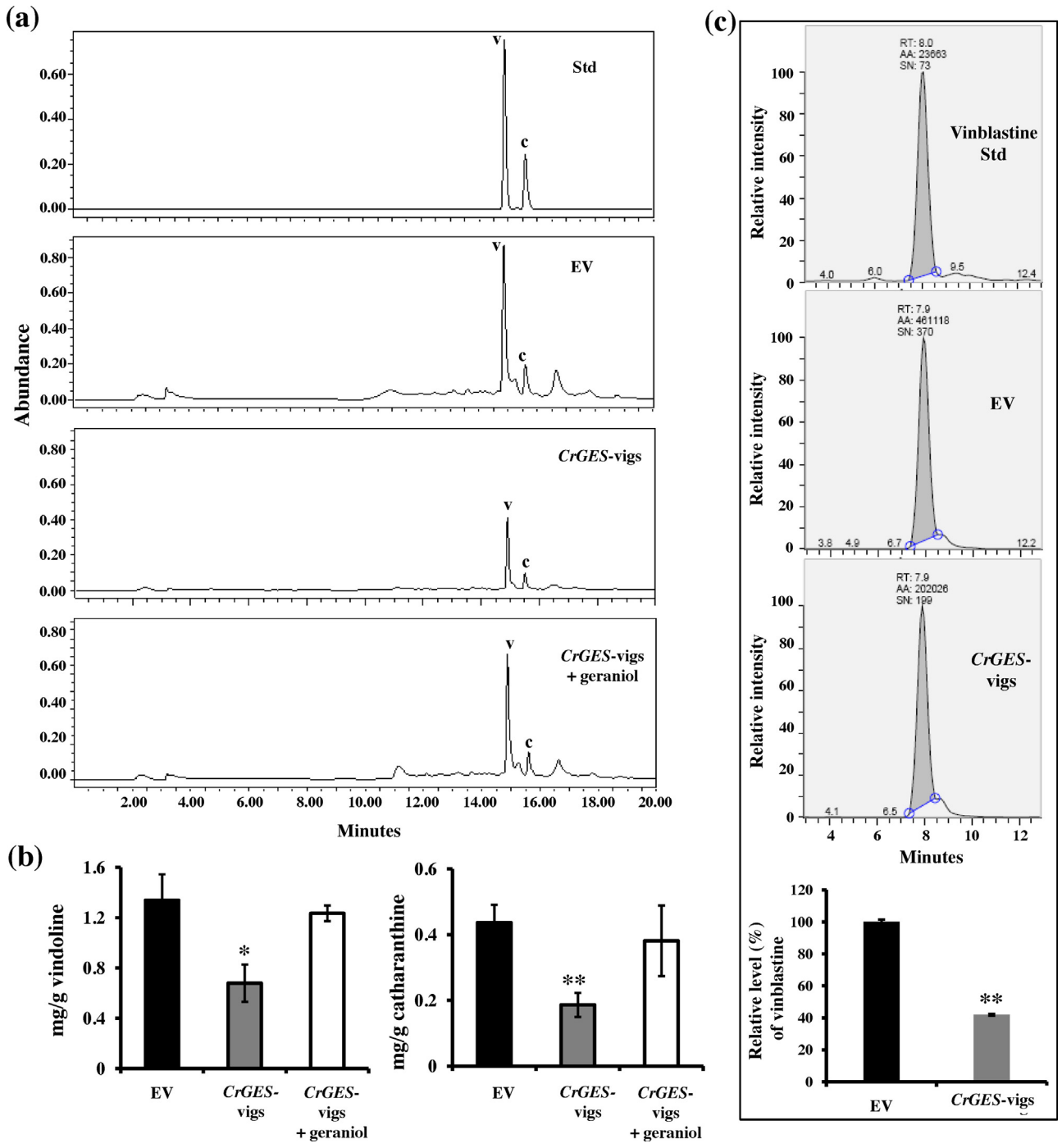

Fig. 5. Effect of *CrGES*-vigs on MIA accumulation in *C. roseus*.
(a) Representative HPLC chromatograms of authentic standards and leaf alkaloids extracted from of EV (top), *CrGES*-vigs (middle) and geraniol-fed *CrGES*-vigs (bottom) leaves. First pair leaves of EV and *CrGES*-vigs leaves were infiltrated with either water or 0.5 mM geraniol. Two days post-infiltration, leaf alkaloids were extracted from 5 mg dry weight and subjected to HPLC analysis and quantification. (b) Relative amounts of vindoline and catharanthine in the leaves of EV (black bar) *CrGES*-vigs (grey bar) and geraniol fed *CrGES*-vigs (white bar) plants. Bars in the figure are mean ± standard error (SE) values of two to five independent experiments. (c) LC–MS/MS analysis and quantification of vinblastine in EV and *CrGES*-vigs leaves. Significant differences at $P < 0.05$ and $P < 0.01$ are indicated by "*" and "**", respectively.

MIA accumulation. The *CrGES* gene was transiently overexpressed in 1st pair of *C. roseus* leaves. Two days after infiltration, leaves were collected and used for transcript and alkaloid analysis. Determination of *GES* transcript levels showed an increase of >3 fold in pBI121:*GES* infiltrated tissues as compared to pBI121 control leaves (Fig. 6a). Subsequent HPLC analysis of MIAs showed increased accumulation of catharanthine (~3-fold) and vindoline (~2.8-fold) in pBI121:*GES* infiltrated samples (Fig. 6b).

3.6. *Expression of* CrGES *correlates with catharanthine and vindoline accumulation in* C. roseus *cultivars*

It has been reported that the MIA content correlates with the expression level of pathway genes [4]. In order to check whether mRNA expression of *CrGES* correlates with the accumulation of catharanthine and vindoline in leaves, we analyzed the transcript levels in 1st leaf pairs of the plant. For this purpose, leaves from low and high alkaloid accumulating *C. roseus* cultivars cv. Nirmal and cv.

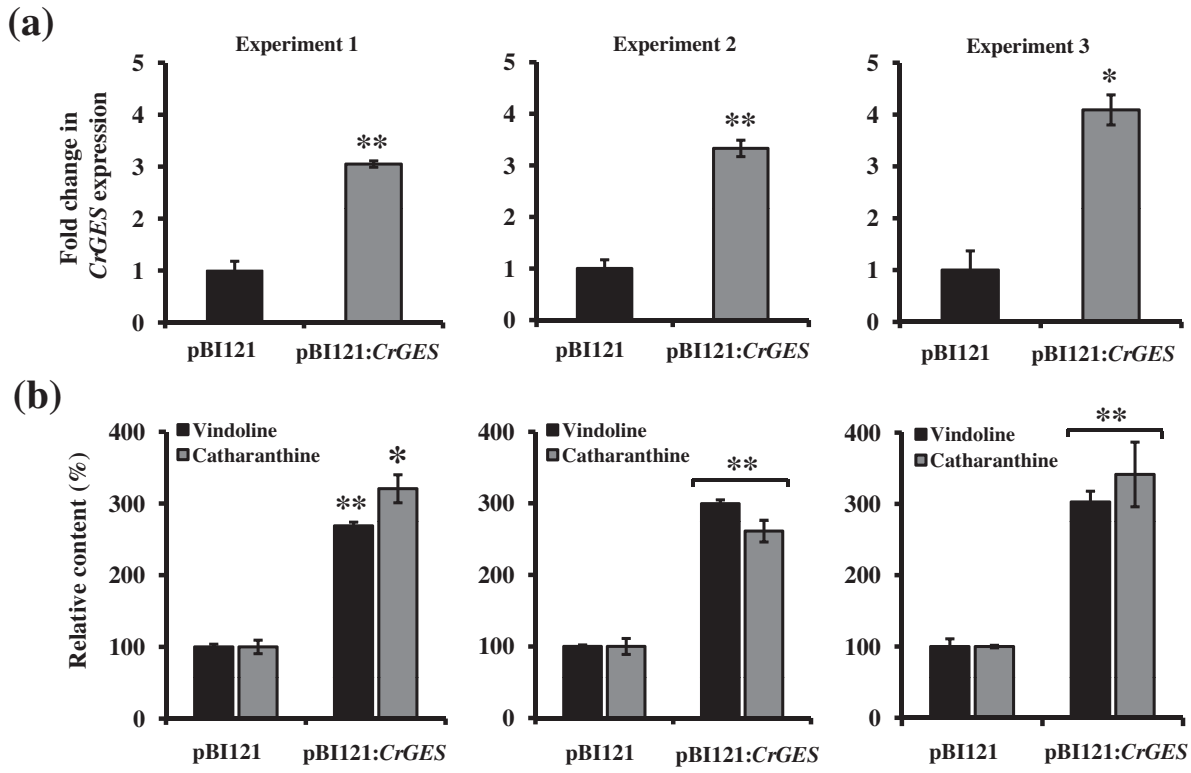

Fig. 6. Effect of transient over-expression of *CrGES* in *C. roseus* leaves.
(a) *CrGES* mRNA expression in *C. roseus* leaves infiltrated with *Agrobacterium* carrying pBI121 vector with only the *CaMV35S* promoter (black bar) or the *GES* gene under the control of the *CaMV35S* promoter (pBI121:*CrGES*; grey bar). *CrGES* mRNA was analyzed by qRT-PCR with *CrRPS9* as a reference gene using comparative *Ct* method. (b) Relative amounts of catharanthine and vindoline in pBI121 (black bar) and pBI121:*CrGES* infiltrated leaves (grey bar). The bars represent mean ± standard error (SE) of three independent experiments. Significant differences at $P < 0.05$ and $P < 0.01$ are represented by "*" and "**", respectively.

Dhawal, respectively [34] were used. Expression analysis showed >3-fold higher level of *CrGES* in cv. Dhawal as compared to cv. Nirmal (Fig. 7a). Analysis of alkaloids clearly exhibited higher content of catharanthine and vindoline in leaves of cv. Dhawal compared to cv. Nirmal (Fig. S4). Subsequent quantification indicated over 3-fold higher accumulation of catharanthine and vindoline in cv. Dhawal, indicating a positive correlation between *CrGES* expression and alkaloid accumulation (Fig. 7b).

3.7. CrGES *has relatively lower expression as compared to upstream and downstream genes*

Increased MIA content upon geraniol feeding and *CrGES* over-expression, and their decrease in *CrGES*-silenced leaves indicated the limitation of endogenous geraniol available for MIA formation in *C. roseus* leaves. To further check whether geraniol accumulation is controlled by transcriptional regulation of *CrGES*, co-expression patterns of *CrGES* transcript levels relative to some of its upstream and downstream genes were measured. The relative expression of MEP pathway genes (*DXS, DXR, MECS* and *HDS*) with respect to *CrGES* ranged from 1.5 to 27 fold with *DXS* exhibiting the maximum expression (Fig. 8). Also, the genes of secoiridoid and indole (*G8O* and *TDC)* pathway showed about 15–43 fold abundance compared to *CrGES* (Fig. 8). Analysis of mRNA expression of MIA pathway genes (*STR, SGD* and *D4H*), as well as the genes encoding transcription factors (ORCA3 and MYC2), also showed higher expression ranging from ~25 to 40 fold relative to *CrGES* (Fig. 8). This indicated that the basal level expression of most pathway genes studied and thus, possibly their corresponding enzymes are remarkably higher as compared to that of *CrGES*.

4. Discussion

The biosynthesis of MIAs depends on precursors from two convergent metabolic pathways: indole and terpenoid. The accumulation of MIAs is thus, dependent on the regulation of the flux through these pathways [21,22,35]. Initial precursor feeding studies in cell suspension cultures indicated inconsistent results with respect to the effect of tryptophan, tryptamine and geraniol on the accumulation of MIAs such as serpentine, ajmalicine, tabersonine, and strictosidine [20,35–37]. However, later studies in cell suspension and hairy roots indicated that geraniol and not tryptophan has a positive effect on accumulation of tabersonine and ajmalicine [21,22]. All these studies were limited to either cell suspension culture or hairy roots. However no such studies have been carried out on the effect of precursor feeding on MIA accumulation in leaves, which are the actual sites for the biosynthesis of monomeric MIA vindoline and dimeric MIAs vinblastine and vincristine. This study was taken up to explore the limitation of terpenoid (geraniol) or indole (tryptophan) branches in leaf MIA formation. While geraniol feeding in leaves increased catharanthine and vindoline levels (Figs. 2a and 5b), tryptophan feeding had no drastic effect (Fig. 2c), indicating the limitation of geraniol availablity for MIA biosynthesis in *C. roseus* leaves. This was also consistent with the observations made in cell culture studies [22]. Unlike geraniol, nerol (an isomer of geraniol) had no effect on alkaloid levels suggesting that the increase in MIAs accumulation upon geraniol feeding is not due to non-specific elicitation. Similar results were also observed by Mor-

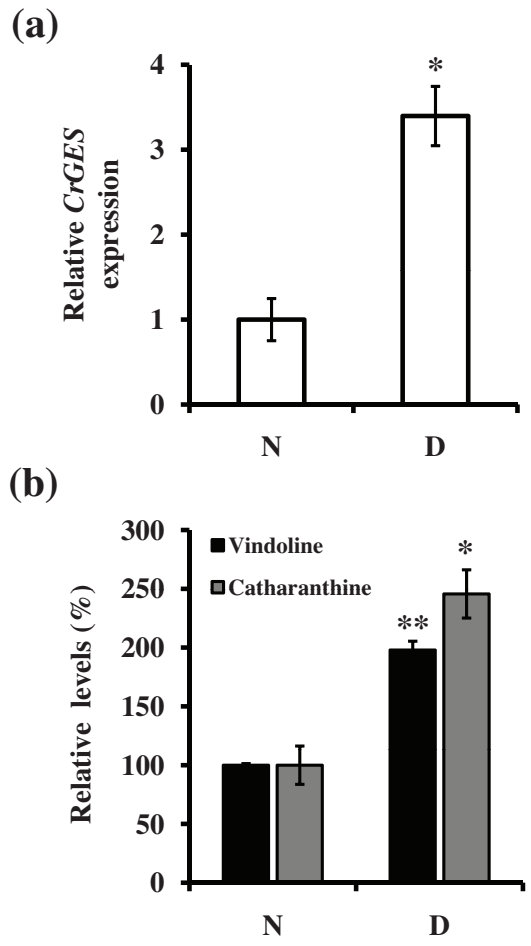

Fig. 7. Relative *CrGES* transcript levels and alkaloids content in different *C. roseus* cultivars.
(a) Relative *CrGES* transcript levels in two cultivars of *C. roseus* cv. Nirmal (N) and cv. Dhawal (D) were performed using total RNA isolated from 1st pair leaves. Transcript levels were determined by qRT-PCR with *CrRPS9* as endogenous control. (b) Relative levels of catharanthine and vindoline content in 1st pair leaves of N and D. The data represented in (a) and (b) are the mean ± standard error (SE) values from two independent experiments with two technical replicates. Significant differences at $P < 0.05$ and $P < 0.01$ are indicated by "*" and "**", respectively.

gan and Shanks [21] on linalool and geraniol feeding on tabersonine accumulation in *C. roseus* hairy roots.

Since feeding of geraniol positively affected the accumulation of vindoline and catharanthine, indicating the *in vivo* limitation of geraniol in the leaf for MIAs biosynthesis, we set out to characterize the *in planta* role of the gene encoding geraniol synthase. Geraniol, formed by the geraniol synthase, a branch point enzyme in the general isoprenoid pathway, serves as the starting point in the formation of iridoid secologanin. Secologanin combines with tryptamine resulting in strictosidine, the central intermediate of MIA biosynthesis [38]. Despite its importance as the initiation step in secoiridoid formation of MIA biosynthesis (Fig. 1), GES has only recently been isolated and enzymatically characterized in *C. roseus* [14]. However, not much is known on the *in planta* role of this gene in MIA biosynthesis. As it is reported that the complex MIA biosynthesis in *C. roseus* is regulated tissue-specifically [39–43], the expression of *CrGES* was analyzed in different tissues. *CrGES* showed highest expression in leaf and shoot (tissues reported to accumulate highest levels of MIAs) followed by flower with least expression in siliques and roots (Fig. 3a). Maximum expression of *CrGES* in leaf and shoot correlated with high accumulation of MIAs in these tissues. Accumulation of *CrGES* transcripts in flowers indicated that it might be involved in floral monoterpene biosynthesis as well as in MIA formation. This was in agreement with RNA *in situ* hybridization studies, which has shown the distribution of *CrGES* in internal phloem associated parenchyma (IPAP) cells of leaves, as well as carpels and stamens of flowers [14]. The gene encoding the small subunit of GPPS (*GPPS.SSU*), which was reported to regulate the formation of GPP in *C. roseus,* also exhibited higher expression in leaf, shoot and flowers [33] indicating co-regulation of GPP and geraniol production. MeJA is known to induce the expression of many genes of secondary metabolic pathways, including the genes of MIA biosynthesis leading to the accumulation of the corresponding metabolites such as alkaloids, terpenoids, flavonoids, and glucosinolates in different plant species [44]. In *C. roseus*, it has been previously reported that several MIA pathway genes such as *GPPS*, *G8O*, *TDC*, *STR*, *D4H, DAT,* and MIA pathway regulators like *ORCA2*, *ORCA3* are induced in response to MeJA resulting in increased alkaloid accumulation [4,33,44,45]. Induction of *CrGES* in response to MeJA in leaf tissue (Fig. 3b) similar to its induction in cell cultures [14] indicated its involvement in MIA biosynthesis.

To further elucidate the *in planta* role of *CrGES*, reverse-genetics VIGS approach was used that has been well validated in *C. roseus* for characterizing several genes lately. Silencing of *CrGES* resulted in ~80% reduction in transcript level (Fig. 4c) with a decrease of ~49% catharanthine and 57% vindoline contents as compared to control (Fig. 5b). Similar level of reduction in catharanthine and vindoline contents ranging from 40 to 60% was observed in VIGS-mediated suppression of genes such as *iridoid synthase* (*IRS*), 7-deoxyloganetic acid glucosyltransferase (*UGT8*), loganic acid *O*-methyltransferase (*LAMT*) and secologanin synthase (*SLS*) [29,46]. Further, measurement of transcript levels of other MIA pathway genes acting downstream (*G8O*, *STR*) of GES and indole pathway gene (*TDC*) as well as the transcriptional regulator (*ORCA3*) showed that there was no effect of *CrGES* silencing on their expression levels (Fig. 4c), indicating that the reduction of catharanthine and vindoline was indeed brought about by *CrGES* silencing, highlighting its importance in MIA biosynthesis. Although there was efficient down-regulation of *CrGES* transcripts (~80%), there was still some degree of catharanthine (~51%) and vindoline (~43%) accumulation (Fig. 5b), which could be possibly formed before the *CrGES* silencing effect took over [26]. Moreover, feeding *GES*-silenced leaves with geraniol resulted in restoration of catharanthine and vindoline levels (Fig. 5b), which provided compelling proof for the involvement of *CrGES* in MIA biosynthesis. Additionally, transient over-expression of the *CrGES* in *C. roseus* leaves resulted in elevated levels of catharanthine and vindoline (Fig. 6), providing additional evidence and thus, supporting VIGS results that *CrGES* plays a crucial role in MIA biosynthesis. Tomato over-expressing basil *GES* exhibited increased geraniol and its derivatives but had reduced lycopene content because of flux redirection [47]. However, metabolic flux analysis of tobacco hairy roots over-expressing *V. officinalis GES* demonstrated an increase of at least an order of magnitude more geraniol without affecting other network fluxes [48]. Thus, over-expression of *GES* in suspension cell/hairy root cultures or at the whole plant level could significantly increase the accumulation of different MIAs, provided concerns of flux diversion from primary metabolism is rationally addressed.

In general, the formation of monoterpenes and sesquiterpenes in several plants is mainly controlled by the expression of corresponding genes [49–51]. Also, in multi-step pathways leading to complex terpenoids, the expression of terpene synthase involved in the initial step influences the accumulation of end products. For example, in tobacco the expression of sesquiterpene synthase regulates the formation and accumulation of defence compound phytoalexin [52]. Similarly in grand fir, a co-ordinated regulation of genes encoding mono-, sesqui- and di-terpene synthases influences the nature of oleoresin formation [53]. In *Artemisia*

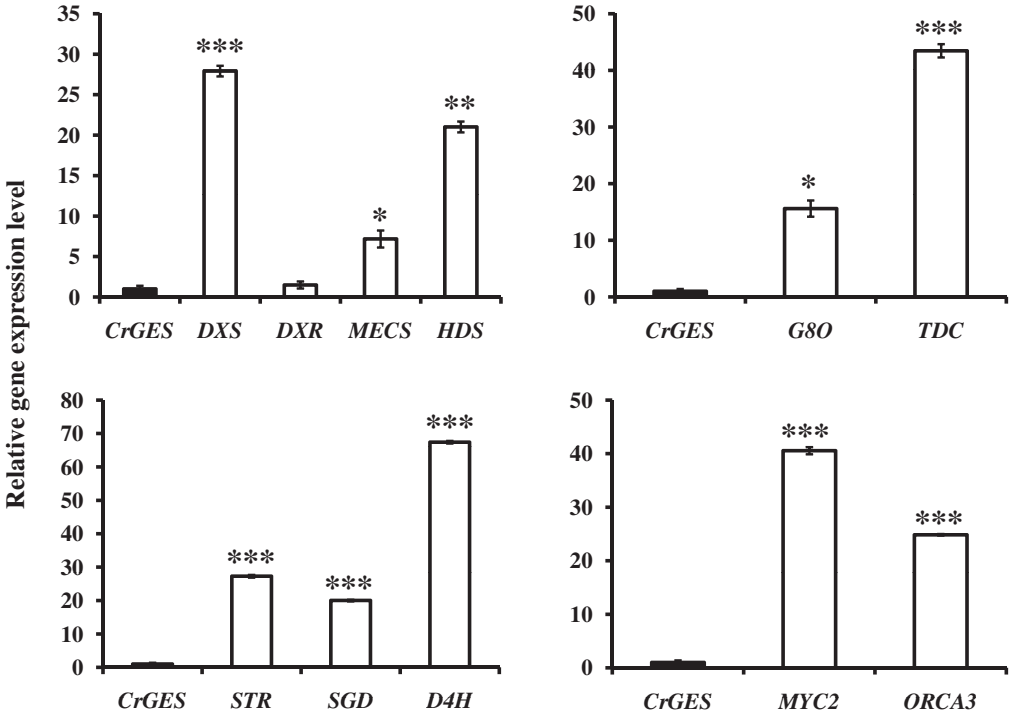

Fig. 8. Comparative transcript levels of pathway genes relative to *CrGES*.
Relative expression of *CrGES* to other pathway genes was determined by qRT-PCR analyses using total RNA extracted from first pair leaves of *C. roseus*. *CrSAND* and *CrN2227* [58,59] were used as reference genes. In all comparisons the mRNA expression of *CrGES* was set to 1. *DXR*, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; *D4H*, deacetoxyvindoline 4-hydroxylase; *DXS*, 1-deoxy-D-xylulose-5-phosphate synthase; *G8O*, geraniol-10-hydroxylase/8-oxidase; *HDS*, (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase; *LMT*, loganic acid methyltransferase; *MECS*, 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase; *MYC1*, bHLH transcription factor that binds the G-box element of the *STR* promoter; *ORCA3*, octadecanoid-responsive *Catharanthus* APETALA-domain protein 3; *SGD*, strictosidine-*O*-β-D-glucosidase; *STR*, strictosidine synthase; *TDC*, tryptophan decarboxylase. The bars represent mean ± standard error (SE) of two independent experiments with two technical replicates of each experiment. Significant differences at $P < 0.05$ and $P < 0.01$ are shown by "*" and "**", respectively.

annua, amorphadiene synthase (ADS) diverts the FPP pool from primary metabolism towards artemisinin biosynthesis [54]. It is reported that alkaloid biosynthesis in plants is regulated primarily at the level of gene expression, which in turn is controlled by the expression of transcription factors [55,56]. Since our results from *CrGES*-VIGS, over-expression and geraniol feeding indicated the limitation of endogenous geraniol for MIA biosynthesis, we determined the expression levels of *CrGES* in low and high MIA accumulating cultivars as well as in relation to other pathway genes. Expression of *CrGES* showed correlation with leaf catharanthine and vindoline content in low (cv. Nirmal) and high (cv. Dhawal) alkaloid accumulators supporting its role in foliar MIA biosynthesis (Fig. 7). A similar trend was also reported for *CYP71D351* (*T16H*), whose expression showed a correlation with vindoline content in different cultivars [57]. Interestingly, all analyzed genes upstream and downstream of GES step showed significantly higher levels of expression compared to the transcript levels of *CrGES* (Fig. 8). Indeed, *in silico* gene expression levels provided as FPKM (Fragments per kilobase per transcript per million mapped reads) values in Medicinal Plant Genomics Resource database (MPGR, http://medicinalplantgenomics.msu.edu) also indicated higher expression of these genes as well as most other genes of MIA pathway (Fig. S5). This suggested that although there is sufficient amount of transcripts and thus, their enzymes of pathway steps downstream and upstream of GES, relatively lower level of geraniol formed by low expressing *CrGES* might act as a limiting factor in the biosynthesis of MIAs.

In conclusion, our results showed the *in planta* limitation of geraniol available for MIA biosynthesis in *C. roseus* leaves that was further validated by molecular characterization of the *CrGES* responsible for geraniol biosynthesis. The spatio-temporal expression of *CrGES* and its correlation to catharanthine and vindoline accumulation indicated its involvement in MIA biosynthesis. VIGS-mediated suppression and over-expression of GES resulting in corresponding reduction and increase in MIA accumulation provided the genetic proof for its involvement in MIA formation. Expression analysis of pathway genes with respect to *CrGES* further provided insights into the crucial role played by GES and its product geraniol in controlling the metabolic flux to secoiridoid pathway and thus, MIA biosynthesis. Our results illustrate that future engineering efforts either in cell culture systems or at the whole plant level should consider this important gene for metabolic modulation for successful improvement in MIA production.

Acknowledgements

This work was supported by grants from Department of Biotechnology, Government of India (grant no. BT/PR6109/AGII/106/857/2012) and BSC-0203 of 12th five year plan of CSIR-CIMAP. DAN thanks Department of Biotechnology, Government of India, for the Ramalingaswami Fellowship (grant no. BT/HRD/35/24/2006). The authors express their sincere gratitude to Prof A.K. Tripathi, Director, CSIR-CIMAP for his keen interest and support. The help rendered by Surjeet Verma and Balakishan Bhukya in ChemDraw and HPLC analysis, respectively, is duly acknowledged. K. Kumar, V. Dwivedi and A. Rai are the recipients of a Research Fellowship from the Council of Scientific and Industrial Research (CSIR, Govt. of India), and University Grants Commission (UGC, Govt. of India), respectively. Authors have no conflict of interest to declare.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2015.07.007

References

[1] R. van Der Heijden, D.I. Jacobs, W. Snoeijer, D. Hallard, R. Verpoorte, The *Catharanthus* alkaloids: pharmacognosy and biotechnology, Curr. Med. Chem. 11 (2004) 607–628.
[2] K. Miettinen, L. Dong, N. Navrot, T. Schneider, V. Burlat, J. Pollier, et al., The seco-iridoid pathway from *Catharanthus roseus*, Nat. Commun. 5 (2014) 3606.
[3] S. Körper, M. Wink, R.H. Fink, Differential effects of alkaloids on sodium currents of isolated single skeletal muscle fibers, FEBS Lett. 436 (1998) 251–255.
[4] L. Zhao, G.W. Sander, J.V. Shanks, Perspectives of the metabolic engineering of terpenoid indole alkaloids in *Catharanthus roseus* hairy roots, Adv. Biochem. Eng. Biotechnol. 134 (2013) 23–54.
[5] X. Ma, S. Panjikar, J. Koepke, E. Loris, J. Stöckigt, The structure of *Rauvolfia serpentina* strictosidine synthase is a novel six-bladed beta-propeller fold in plant proteins, Plant Cell 18 (2006) 907–920.
[6] S.E. O'Connor, J.J. Maresh, Chemistry and biology of monoterpene indole alkaloid biosynthesis, Nat. Prod. Rep. 23 (2006) 532–547.
[7] P.J. Facchini, V. De Luca, Opium poppy and Madagascar periwinkle: model non-model systems to investigate alkaloid biosynthesis in plants, Plant J. 54 (2008) 763–784.
[8] N. Dudareva, F. Negre, D.A. Nagegowda, I. Orlova, Plant volatiles: recent advances and future perspectives, CRC. Crit. Rev. Plant Sci. 25 (2006) 417–440.
[9] D. Tholl, Terpene synthases and the regulation, diversity and biological roles of terpene metabolism, Curr. Opin. Plant Biol. 9 (2006) 297–304.
[10] Y. Iijima, D.R. Gang, E. Fridman, E. Lewinsohn, E. Pichersky, Characterization of geraniol synthase from the peltate glands of sweet basil, Plant Physiol. 134 (2004) 370–379.
[11] T. Yang, J. Li, H.-X. Wang, Y. Zeng, A geraniol-synthase gene from *Cinnamomum tenuipilum*, Phytochemistry 66 (2005) 285–293.
[12] M. Ito, G. Honda, Geraniol synthases from perilla and their taxonomical significance, Phytochemistry 68 (2007) 446–453.
[13] L. Dong, K. Miettinen, M. Goedbloed, F.W.A. Verstappen, A. Voster, M.A. Jongsma, et al., Characterization of two geraniol synthases from *Valeriana officinalis* and *Lippia dulcis*: similar activity but difference in subcellular localization, Metab. Eng. 20 (2013) 198–211.
[14] A.J. Simkin, K. Miettinen, P. Claudel, V. Burlat, G. Guirimand, V. Courdavault, et al., Characterization of the plastidial geraniol synthase from Madagascar periwinkle which initiates the monoterpenoid branch of the alkaloid pathway in internal phloem associated parenchyma, Phytochemistry 85 (2013) 36–43.
[15] S. Brown, M. Clastre, V. Courdavault, V.S.E. O'Connor, De novo production of the plant-derived alkaloid strictosidine in yeast, Proc. Natl. Acad. Sci. U. S. A. 112 (2015) 3205–3210.
[16] Y. Qu, M.L. Easson, J. Froese, R. Simionescu, T. Hudlicky, V. De Luca, Completion of the seven-step pathway from tabersonine to the anticancer drug precursorvindoline and its assembly in yeast, Proc. Natl. Acad. Sci. U. S. A. (2015), http://dx.doi.org/10.1073/pnas.1501821112/-/DCSupplemental
[17] G. Döller, A.W. Alfermann, E. Reinhard, Production of indole alkaloids in tissue cultures of *Catharanthus roseus* (author's transl)], Planta Med. 30 (1976) 14–20.
[18] M.H. Zenk, H. El-Shagi, H. Arens, J. Stockigt, E.W. Weiler, B. Deus, Formation of the indole alkaloids serpentine and ajamalicine in cell suspension culture of Catharanthus roseus, in: W. Bare, E. Reinhard, M.H. Zenk (Eds.), Plant Tissue Culture and Its Bio-technological Application, Springer-Verlag, Berlin, 1997, pp. 22–44.
[19] J.M. Mérillon, P. Doireau, A. Guillot, J.C. Chénieux, M. Rideau, Indole alkaloid accumulation and tryptophan decarboxylase activity in *Catharanthus roseus* cells cultured in three different media, Plant Cell Rep. 5 (1986) 23–26.
[20] P.J. Facchini, F. DiCosmo, Secondary metabolite biosynthesis in cultured cells of *Catharanthus roseus* (L.) G. Don immobilized by adhesion to glass fibres, Appl. Microbiol. Biotechnol. 35 (1991) 382–392.
[21] J.A. Morgan, J.V. Shanks, Determination of metabolic rate-limitations by precursor feeding in *Catharanthus roseus* hairy root cultures, J. Biotechnol. 79 (2000) 137–145.
[22] C.W.T. Lee-Parsons, A.J. Royce, Precursor limitations in methyl jasmonate-induced *Catharanthus roseus* cell cultures, Plant Cell Rep. 25 (2006) 607–612.
[23] S. Whitmer, C. Canel, D. Hallard, C. Gonçalves, R. Verpoorte, Influence of precursor availability on alkaloid accumulation by transgenic cell line of *Catharanthus roseus*, Plant Physiol. 116 (1998) 853–857.
[24] Y. Liu, M. Schiff, S.P. Dinesh-Kumar, Virus-induced gene silencing in tomato, Plant J. 31 (2002) 777–786.
[25] R. Höfgen, L. Willmitzer, Storage of competent cells for *Agrobacterium* transformation, Nucleic Acids Res. 16 (1988) 9877.
[26] D.K. Liscombe, S.E. O'Connor, A virus-induced gene silencing approach to understanding alkaloid metabolism in *Catharanthus roseus*, Phytochemistry 72 (2011) 1969–1977.
[27] M. de Lourdes Miranda-Ham, I. Islas-Flores, A.F. Vázquez-Flota, Accumulation of monoterpenoid indole alkaloids in periwinkle seedlings (*Catharanthus roseus*) as a model for the study of plant-environment interactions, Biochem. Mol. Biol. Educ. 35 (2007) 206–210.
[28] N. Suttipanta, S. Pattanaik, M. Kulshrestha, B. Patra, S.K. Singh, L. Yuan, The transcription factor CrWRKY1 positively regulates the terpenoid indole alkaloid biosynthesis in *Catharanthus roseus*, Plant Physiol. 157 (2011) 2081–2093.
[29] K. Asada, V. Salim, S. Masada-Atsumi, E. Edmunds, M. Nagatoshi, K. Terasaka, et al., A 7-deoxyloganetic acid glucosyltransferase contributes a key step in secologanin biosynthesis in Madagascar periwinkle, Plant Cell 25 (2013) 4123–4134.
[30] T.M. Burch-Smith, J.C. Anderson, G.B. Martin, S.P. Dinesh-kumar, Application and advantage of virus-induced gene silencing for gene function studies in plants, Plant J. 39 (2004) 734–746.
[31] S. Di Fiore, V. Hoppmann, R. Fischer, S. Schillberg, Transient gene expression of recombinant terpenoid indole alkaloid enzymes in *Catharanthus roseus* leaves, Plant Mol. Biol. Rep. 22 (2012) 15–22.
[32] S.K. Raina, D.P. Wankhede, M. Jaggi, P. Singh, S.K. Jalmi, B. Raghuram, et al., CrMPK3, a mitogen activated protein kinase from *Catharanthus roseus* and its possible role in stress induced biosynthesis of monoterpenoid indole alkaloids, BMC Plant Biol. 12 (2012) 134.
[33] A. Rai, S.S. Smita, A.K. Singh, K. Shanker, D.A. Nagegowda, Heteromeric and homomeric geranyl diphosphate synthases from *Catharanthus roseus* and their role in monoterpene indole alkaloid biosynthesis, Mol. Plant 6 (2013) 1531–1549.
[34] A.K. Shukla, Molecular studies on biosynthesis of shoot alkaloids in *Catharanthus roseus* (L.) G. Don, in: PhD Thesis, Department of Biochemistry, University of Lucknow, India, 2005.
[35] P.R. Moreno, R. van der Heijden, R. Verpoorte, Effect of terpenoid precursor feeding and elicitation on formation of indole alkaloids in cell suspension cultures of *Catharanthus roseus*, Plant Cell Rep. 12 (1993) 702–705.
[36] R.J. Krueger, D.P. Carew, *Catharanthus roseus* tissue culture: the effects of precursors on growth and alkaloids production, Lloydia 41 (1978) 327–331.
[37] A. Contin, R. van Der Heijden, A.W.M. Lefeber, R. Verpoorte, The iridoid glucoside secologanin is derived from the novel triose phosphate: pyruvate pathways in *Catharanthus roseus* cell culture, FEBS Lett. 434 (1998) 413–416.
[38] G. Guirimand, V. Courdavault, A. Mahroug, S. Guihur, A. Guihur, N. Blanc, N. Giglioli-Guivarch, B. St-Pierre, V. Burlat, Strictosidine activation in Apocynaceae: towards a nuclear time bomb? BMC Plant Biol. 10 (2010) 147.
[39] A.K. Shukla, A.K. Shasany, M.M. Gupta, S.P.S. Khanuja, Transcriptome analysis in Catharanthus roseus leaves and roots for comparative terpenoid indole alkaloid profiles, J. Exp. Bot. 57 (2006) 3921–3932.
[40] B. St-Pierre, F. Vazquez-Flota, V. De Luca, Multicellular compartmentation of catharanthus roseus alkaloid biosynthesis predicts intercellular translocation of a pathway intermediate, Plant Cell. 11 (1999) 887–900.
[41] V. De Luca, B. St Pierre, The cell and developmental biology of alkaloid biosynthesis, Trends Plant Sci. 5 (2000) 168–173.
[42] P.J. Facchini, Alkaloids biosynthesis in plants: biochemistry, cell biology, molecular regulation, and metabolic engineering applications, Annu. Rev. Plant Physiol Plant Mol. Biol. 52 (2001) 29–66.
[43] J. Roepke, V. Salim, M. Wu, A.M.K. Thamm, J. Murata, K. Ploss, et al., Vinca drug components accumulate exclusively in leaf exudates of Madagascar periwinkle, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 15287–15292.
[44] J. Memelink, R. Verpoorte, J.W. Kijne, ORCAnization of jasmonate-responsive gene expression in alkaloid metabolism, Trends Plant Sci. 6 (2001) 212–219.
[45] L. Van der Fits, J. Memelink, ORCA3, a jasmonate-responsive transcriptional regulator of plant primary and secondary metabolism, Science 289 (2000) 295–297.
[46] F. Geu-Flores, N.H. Sherden, V. Courdavault, V. Burlat, W.S. Glenn, C. Wu, et al., An alternative route to cyclic terpenes by reductive cyclization in iridoid biosynthesis, Nature 492 (2012) 138–142.
[47] R. Davidovich-Rikanati, Y. Sitrit, Y. Tadmor, Y. Iijima, N. Bilenko, E. Bar, et al., Enrichment of tomato flavor by diversion of the early plastidial terpenoid pathway, Nat. Biotechnol. 25 (2007) 899–901.
[48] S.K. Masakapalli, A. Ritala, L. Dong, A.R. van der Krol, K.-M. Oksman-Caldentey, R.G. Ratcliffe, et al., Metabolic flux phenotype of tobacco hairy roots engineered for increased geraniol production, Phytochemistry 99 (2014) 73–85.
[49] N. Dudareva, D. Martin, C.M. Kish, N. Kolosova, N. Gorenstein, J. Fäldt, et al., (E)-beta-ocimene and myrcene synthase genes of floral scent biosynthesis in snapdragon: function and expression of three terpene synthase genes of a new terpene synthase subfamily, Plant Cell 15 (2003) 1227–1241.
[50] D.A. Nagegowda, M. Gutensohn, C.G. Wilkerson, N. Dudareva, Two nearly identical terpene synthases catalyze the formation of nerolidol and linalool in snapdragon flowers, Plant J. 55 (2008) 224–239.
[51] D.A. Nagegowda, Plant volatile terpenoid metabolism: biosynthetic genes, transcriptional regulation and subcellular compartmentation, FEBS Lett. 584 (2010) 2965–2973.
[52] M. Zook, T. Hohn, A. Bonnen, J. Tsuji, R. Hammerschmidt, Characterization of novel sesquiterpenoid biosynthesis in tobacco expressing a fungal sesquiterpene synthase, Plant Physiol. 112 (1996) 311–318.
[53] C. Steele, S. Katoh, J. Bohlmann, R. Croteau, Regulation of oleoresinosis in grand fir (*Abies grandis*). Differential transcriptional control of monoterpene, sesquiterpene, and diterpene synthase genes in response to wounding, Plant Physiol. 116 (1998) 1497–1504.

[54] P.R. Arsenault, D. Vail, K.K. Wobbe, K. Erickson, P.J. Weathers, Reproductive development modulates gene expression and metabolite levels with possible feedback inhibition of artemisinin in *Artemisia annua*, Plant Physiol. 154 (2010) 958–968.
[55] V. De Luca, P. Laflamme, The expanding universe of alkaloid biosynthesis, Curr. Opin. Plant Biol. 4 (2001) 225–233.
[56] D. Vom Endt, J.W. Kijne, J. Memelink, Transcription factors controlling plant secondary metabolism: what regulates the regulators, Phytochemistry 61 (2002) 107–114.
[57] S. Besseau, F. Kellner, A. Lanoue, A.M.K. Thamm, V. Salim, B. Schneider, et al., A pair of tabersonine 16-hydroxylases initiates the synthesis of vindoline in an organ-dependent manner in *Catharanthus roseus*, Plant Physiol. 163 (2013) 1792–1803.
[58] J. Pollier, R. Vander Bossche, H. Rischer, A. Goossens, Selection and validation of reference genes for transcript normalization in gene expression studies in *Catharanthus roseus*, Plant Physiol. Biochem. 83 (2014) 20–25.
[59] A. Van Moerkercke, P. Steensma, F. Schweizer, J. Pollier, I. Gariboldi, et al., The bHLH transcription factor BIS1 controls the iridoid branch of the monoterpenoid indole alkaloid pathway in *Catharanthus roseus*, Proc. Natl. Acad. Sci. U. S. A. (2015), http://dx.doi.org/10.1073/pnas.1504951112.