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

Krishna Kumar[a,b], Sarma Rajeev Kumar[a,b], Varun Dwivedi[a,b], Avanish Rai[a,b], Ashutosh K. Shukla[b], Karuna Shanker[c], Dinesh A. Nagegowda[a,b,*]

[a] CSIR-Central Institute of Medicinal and Aromatic Plants Research Centre, Bengaluru 560065, India
[b] Biotechnology Division, CSIR-Central Institute of Medicinal and Aromatic Plants, P.O. CIMAP, Lucknow 226015, India
[c] Analytical Chemistry Department, CSIR-Central Institute of Medicinal and Aromatic Plants, P.O. CIMAP, Lucknow 226015, India

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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.

* Corresponding author at: CSIR-Central Institute of Medicinal and Aromatic Plants Research Centre, Allalasandra, GKVK Post, Bengaluru 560065, India. Fax: +91 80 28564707.
E-mail address: da.nagegowda@cimap.res.in (D.A. Nagegowda).

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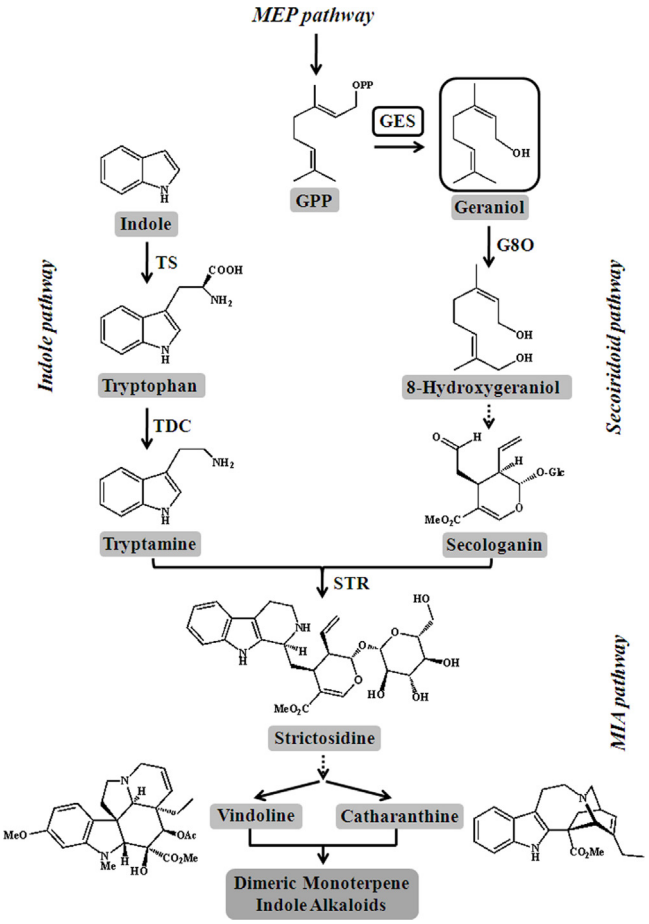

Fig. 1. Simplified view of MIA biosynthesis in *C. roseus*.
Full and dashed arrows indicate single and multiple enzymatic steps, respectively. The monoterpene branching step, GES and its product geraniol are boxed. The name of enzyme involved in each step is indicated on the right side of arrow. The 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