Terpene Moiety Enhancement by Overexpression of Geranyl(geranyl) Diphosphate Synthase and Geraniol Synthase Elevates Monomeric and Dimeric Monoterpene Indole Alkaloids in Transgenic Catharanthus roseus

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Catharanthus roseus is the sole source of two of the most important anticancer monoterpenoid indole alkaloids (MIAs), vinblastine and vincristine and their precursors, vindoline and catharanthine. The MIAs are produced from the condensation of precursors derived from indole and terpene secoiridoid pathways. It has been previously reported that the terpene moiety limits MIA biosynthesis in C. roseus. Here, to overcome this limitation and enhance MIAs levels in C. roseus, bifunctional geranyl(geranyl) diphosphate synthase [G(G)PPS] and geraniol synthase (GES) that provide precursors for early steps of terpene moiety (secologanin) formation, were overexpressed transiently by agroinfiltration and stably by Agrobacterium-mediated transformation. Both transient and stable overexpression of G(G)PPS and co-expression of G(G)PPS+GES significantly enhanced the accumulation of secologanin, which in turn elevated the levels of monomeric MIAs. In addition, transgenic C. roseus plants exhibited increased levels of root alkaloid ajmalicine. The dimeric alkaloid vinblastine was enhanced only in G(G)PPS but not in G(G)PPS+GES transgenic lines that correlated with transcript levels of peroxidase-1 (PRX1) involved in coupling of vindoline and catharanthine into 3′,4′-anhydrovinblastine, the immediate precursor of vinblastine. Moreover, first generation (T1) lines exhibited comparable transcript and metabolite levels to that of T0 lines. In addition, transgenic lines displayed normal growth similar to wild-type plants indicating that the bifunctional G(G)PPS enhanced flux toward both primary and secondary metabolism. These results revealed that improved availability of early precursors for terpene moiety biosynthesis enhanced production of MIAs in C. roseus at the whole plant level. This is the first report demonstrating enhanced accumulation of monomeric and dimeric MIAs including root MIA ajmalicine in C. roseus through transgenic approaches.

Keywords: Catharanthus roseus, geraniol synthase, geranyl(geranyl) diphosphate synthase, overexpression, monoterpenoid indole alkaloids, transgenic plant
INTRODUCTION

Catharanthus roseus (Madagascar Periwinkle) is the best-characterized monoterpene indole alkaloids (MIAs)-producing plant species. To date, C. roseus remains the only natural source of two medicinally valuable dimeric MIAs vinblastine and vincristine, and their monomeric precursors vindoline and catharanthine (Miettinen et al., 2014; Kellner et al., 2015). While leaf specific dimeric alkaloids vinblastine and vincristine are used either directly or as derivatives in cancer chemotherapy, roots accumulate monomeric alkaloids ajmalicine and serpentine which are used as anti-hypertensive agents (Zhao et al., 2013). Coupling of the monomeric precursors vindoline and catharanthine to α-3′,4′-anhydrovinblastine and its subsequent conversion results in the formation of dimeric vinblastine and vincristine (Costa et al., 2008). Extremely low in planta accumulation of dimeric alkaloids makes them highly expensive. Approximately 500 kg of dry leaves is required to isolate 1 g of vinblastine for pharmaceutical production (Noble, 1990). Moreover, total chemical synthesis of dimeric alkaloids is economically not viable due to their complex structures (Kuboyama et al., 2004; van Der Heijden et al., 2004; Ishikawa et al., 2008). Furthermore, improving the production of monomeric vindoline and dimeric alkaloids in cell suspension and hairy root cultures is challenging as they lack the required level of cellular and tissue differentiation essential for the expression of entire MIAs pathway genes, especially the genes involved in vindoline biosynthesis (Zarate and Verpoorte, 2007; Guirimand et al., 2009). Such a scenario calls for genetic transformation of C. roseus for improvement of MIAs production at the whole plant level.

To date, genetic transformation in C. roseus has been mostly confined to hairy roots and cell cultures (Choi et al., 2004; Peebles et al., 2006; Jaggi et al., 2011; Sun et al., 2017). Moreover, transgenic C. roseus cell suspension cultures do not produce alkaloids in a stable manner and their ability to accumulate MIAs declines by prolonged subculture (Whitmer et al., 2003). Not many efforts have been made with respect to metabolic engineering of C. roseus plants, as they are hard-to-transform owing to their highly recalcitrant nature for genetic transformation. Lately, some reports have demonstrated the generation of transgenic C. roseus through Agrobacterium tumefaciens-mediated transformation (Verma and Mathur, 2011b; Pan et al., 2012; Wang Q. et al., 2012). Direct shoot bud organogenesis and transformation were achieved using pre-plasmolyzed leaf explants and β-glucuronidase (GUS) expression was confirmed in transgenic plants (Verma and Mathur, 2011b). However, there are only few reports pertaining to development of transgenic C. roseus overexpressing MIA pathway genes or regulators (Pan et al., 2012; Wang Q. et al., 2012). It was shown that transgenic overexpression of deacetylvinindole-4-O-acetyltransferase (DAT) in C. roseus plants resulted in improved accumulation of vindoline (Wang Q. et al., 2012). Another study from the same group reported enhanced production of ajmalicine, catharanthine and vindoline in transgenic C. roseus overexpressing geraniol 10-hydroxylase (G10H) and the transcriptional regulator Octadecanoid-derivative Responsive Catharanthus AP2-domain (ORCA3) (Pan et al., 2012; Wang Q. et al., 2012).

Monoterpene indole alkaloids are derived from the central intermediate strictosidine, which is formed by the condensation of indole pathway derived tryptamine and monoterpene iridoid precursor secologanin in a reaction catalyzed by the enzyme strictosidine synthase (STR) (Figure 1). While the indole moiety tryptamine is formed from tryptophan, the iridoid biosynthesis starts from the monoterpene geraniol. Geraniol is formed from geranyl diphosphate (GPP) by the action of a terpene synthase, geraniol synthase (GES). GPP, the universal precursor of monoterpene, is produced by the condensation of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) by GPP synthase (GPPS) (Dudareva et al., 2006; Nagegowda, 2010). Geraniol is further oxidized by geraniol-10-hydroxylase/8-oxidase (G8O) to 8-hydroxygeraniol, which is then converted to loganin and finally to secologanin by multiple enzymatic steps (Miettinen et al., 2014). In plants, GPPS functions at the branch point of general and specialized metabolism and regulates flux to monoterpene biosynthesis (Orlova et al., 2009; Nagegowda, 2010). In tobacco, overexpression of snapdragon GPPS small subunit (GPPS.SSU) resulted in an increased monoterpene emission in leaves. However, plants were dwarfed and exhibited strong leaf chlorotic symptoms together with increased light sensitivity due to reduction in the level of primary metabolites (Orlova et al., 2009). In another study, co-expression of peppermint GPS.SSU with four different monoterpene synthases boosted monoterpene production in transgenic tobacco without negatively affecting plant growth (Yin et al., 2017). These studies indicate that, overexpression of either GPPS alone or co-expression of GPPS with a monoterpene synthase could enhance the production of monoterpenes.

In C. roseus, the heteromeric GPPS consisting of an enzymatic large subunit (LSU) and an inactive small subunit (SSU) is involved in providing GPP required for MIA biosynthesis. The LSU is a bifunctional G(G)PP synthase [G(G)PPS] that produces both GPP and GGPP in a ∼2:1 ratio (Rai et al., 2013). Further, GPP is utilized by the monoterpene synthase GES, which is transcriptionally regulated and its transient overexpression in leaves enhanced monomeric alkaloids in C. roseus (Kumar et al., 2015). It has been reported in cell cultures that the terpene moiety is limiting for the biosynthesis of MIAs (Peebles et al., 2006). Moreover, we have previously shown by feeding studies that geraniol and not tryptophan is limiting for the biosynthesis of vindoline and catharanthine in C. roseus leaves (Kumar et al., 2015). Thus, enhanced availability of early secoiridoid pathway precursors (GPP and geraniol) could push the flux toward secologanin, which in turn could enhance the accumulation of end products (monomeric/dimeric alkaloids). In this work, we overexpressed a bifunctional G(G)PPS, and co-expressed G(G)PPS and GES in transgenic C. roseus plants that were generated through Agrobacterium-mediated transformation. Transgenic plants accumulated enhanced secologanin, vindoline, catharanthine, ajmalicine and vinblastine without any negative effects on plant growth, thus demonstrating the importance of metabolic engineering at the whole plant level for improvement of monomeric and dimeric MIAs production in C. roseus.
FIGURE 1 | Simplified version of monoterpene indole alkaloids (MIAs) biosynthetic pathway in C. roseus. Full and dashed arrows indicate single and multiple enzymatic steps, respectively. The genes used to generate transgenic plants, geranyl(geranyl) diphosphate synthase [G(G)PPS] and geraniol synthase (GES), are boxed. Structures of analyzed metabolites in this study are shown in gray highlighted boxes. DMAPP, dimethylallyl diphosphate; G8O, geraniol-10-hydroxylase/8-oxidase; GPP, geranyl diphosphate; IPP, isopentenyl diphosphate; PRX1, peroxidase 1; SLS, secologanin synthase; STR, strictosidine synthase; TDC, tryptophan decarboxylase.
MATERIALS AND METHODS

Generation of Plant Overexpression Vectors

For generation of G(G)PPS plant overexpression construct, the open reading frame corresponding to 1,152 bp was PCR-amplified from leaf cDNA of C. roseus (cv. Dhawal) using full length gene-specific primers (Supplementary Table S1) containing XbaI and SacI sites. The amplified coding regions were cloned into the pJET1.2 vector and nucleotide sequences were confirmed by Sanger sequencing. Resulting pJET constructs were restriction digested and G(G)PPS was sub-cloned into XbaI and SacI sites of pBI121 binary vector under the control of 35S promoter of Cauliflower mosaic virus (CaMV) to form pBI121::G(G)PPS construct (Supplementary Figure S1). Agrobacterium tumefaciens strain GV3101 was transformed with pBI121 empty vector and pBI121::G(G)PPS construct by freeze-thaw method. For GES overexpression, the plant overexpression construct pBI121::GES reported in Kumar et al. (2015) was used.

Transient Overexpression in C. roseus

Transient overexpression was performed following the method described in Kumar et al. (2015). Briefly, overnight grown Agrobacteria cultures were pelleted and resuspended in infiltration buffer (50 mM MES pH 5.6, 2 mM Na3PO4, 0.5% glucose and 100 µM acetylsyringone) to a final OD600 of 0.2. The Agrobacteria suspension was incubated at 28°C for 4 h. Infiltration was performed into the first pair of leaves of 3-week-old C. roseus plants using a needle-less syringe. Post-infiltration, plants were covered and maintained in the dark for 48 h. Infiltrated leaves were harvested and used for subsequent gene expression and metabolite analyses.

Generation of Transgenic C. roseus Plants

C. roseus (cv. Dhawal) seeds were surface sterilized using 4% sodium hypochlorite and inoculated in half strength MS (Murashige and Skoog, 1962) medium. In vitro grown hypocotyl and nodal explants were used for generation of G(G)PPS and G(G)PPS+GES transgenic lines, respectively. While G(G)PPS transformants were generated by indirect regeneration using hypocotyl explants according to Wang Q. et al. (2012), plants co-expressing G(G)PPS+GES were generated by direct regeneration using nodal explants following the method reported in Verma and Mathur (2011a).

For generating transgenic plants through indirect regeneration using hypocotyl explants, MS basal medium was supplemented with 250 mg/L proline and 150 mg/L casein hydrolysate (MSCP) with appropriate hormones as follows. Hypocotyl explants were precultured in MSCP1 medium [MSCP with 1.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 1.0 mg/L α-naphthalene acetic acid (NAA) and 0.1 mg/L zeatin] for 3 days. After co-cultivation with Agrobacteria cultures, explants were transferred to MSCP1 selection medium for a week and subsequently transferred to MSCP2 medium [MSCP with 5.0 mg/L 6-benzyl adenine (BA), 0.5 mg/L NAA] for callus induction. Finally, the initiated shoots were transferred to MSCP3 shoot regeneration medium [MSCP with 1.75 mg/L BA, 0.55 mg/L indole-3-acetic acid (IAA)].

For direct regeneration, nodal explants (1–2 cm) were excised from 1-month-old in vitro grown C. roseus plants. Subsequently, nodal explants were co-cultivated with 1:1 mixture of Agrobacteria harboring pBI121::G(G)PPS and pBI121::GES (OD600 0.4–0.6) for 10 min. The explants were then blot-dried and inoculated into MS-BNT medium containing 1 mg/L BA, 0.1 mg/L NAA and 400 mg/L thiamine HCl. After 3 days of co-cultivation in dark, explants were transferred to fresh MS-BNT medium containing kanamycin and carbenicillin. For shoot proliferation, explants were continued to be cultured in the same medium by subculturing every 10 days. After five rounds of subculturing, well developed shoots were transferred to half strength MS medium for rooting. In both cases, our own modifications were made for rooting as outlined below. After obtaining putative transgenic shoots, they were excised and inoculated initially in rooting media consisting of half strength MS with 2 mg/L indole-3-butyric acid (IBA) and 300 mg/L carbenicillin until root initiation. Later, these plants were transferred to half strength MS with 2.4 µM IBA and 300 mg/L carbenicillin. Plants with well developed roots were transplanted to pots containing 1:1 sterile soilrite:vermicompost mix, and transferred to glass house after acclimation.

PCR Confirmation of Transgenic Lines and Quantitative Reverse Transcriptase-PCR (qRT-PCR) Analysis

Genomic DNA was isolated from wild type (WT) controls and putative transgenic lines following the protocol reported by Doyle and Doyle (1990). Briefly, leaf disks were ground with preheated extraction buffer (1.4 M NaCl, 20 mM EDTA, 100 mM Tris HCl, 0.2% β-Mercaptoethanol, 2% PVP, and 2% CTAB) and incubated at 65°C for 1 h with intermittent shaking. An equal volume of 24:1 chloroform:isoamyl alcohol was added to separate the aqueous phase. The DNA was precipitated by adding 0.6 volume of ice-cold isopropanol to the aqueous phase and incubated overnight at −20°C. Precipitated genomic DNA was then washed with 70% ethanol and finally resuspended in milliQ water. Transgenic lines were confirmed by PCR screening using appropriate primers. In the case of G(G)PPS lines, 35S promoter-specific forward and G(G)PPS-specific reverse primers were used. For screening G(G)PPS+GES lines, 35S/nptII promoter-specific forward and 35S/G(G)PPS/GES/nptII specific reverse primers were used (Supplementary Table S1). RNA isolation, cDNA synthesis and qRT-PCR were performed in transgenic lines following the procedure reported in Rai et al. (2013) and Kumar et al. (2015). Total RNA was extracted from 100 mg leaf tissue using the Spectrum™ Plant Total RNA kit (Sigma-Aldrich, United States) following manufacturer’s instructions. To remove contaminating genomic DNA, on-column DNase digestion was performed with DNase I (Sigma-Aldrich, St. Louis, MO, United States). Total RNA (2 μg) was used for first strand cDNA synthesis with
random hexamer primers using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, United States) as per manufacturer’s instructions. qRT-PCR was performed with a linear range of cDNA using StepOne Real-Time PCR System (Applied Biosystems, United States) and expression of transcripts were normalized using N227 (Applied Biosystems, United States) and expression of transcripts in gene expression were analyzed by the comparative cycle threshold (CT) method. Relative quantification was carried out by calculating CT to determine fold difference in gene expression [\( \Delta \Delta CT \)]. The relative level was determined as \( 2^{-\Delta \Delta CT} \). All experiments were repeated using three biological replicates with three technical replicates and data were analyzed statistically as mentioned below.

Alkaloid Extraction, Analysis, and Quantification
Secologanin, vindoline, catharanthine, ajmalicine, and vinblastine were quantified using High Performance Liquid Chromatography (HPLC) having Photodiode Array (PDA) detector. HPLC (Model: SCL-10AVP, Shimadzu, Japan) equipped with C18 symmetry reverse phase column (5 µm, 250 mm × 4.6 mm, Waters, Milford, MA, United States) was used for all analyses. Secologanin was extracted and quantified according to Tikhomiroff and Jolicoeur (2002) with minor modifications. Fresh leaves (50 mg) from 2-month-old plants were extracted in 1.5 ml methanol for 60 min in a sonication bath. The extract was centrifuged and the supernatant was decolorized by adding activated charcoal. Subsequently, the supernatant was transferred to a fresh tube and evaporated to dryness. The dried residue was dissolved in 20 µl methanol and used for HPLC analysis. Mobile phase for HPLC consisted of 15:85 (v/v) mixture of gradient grade acetonitrile-phosphoric acid (0.1 M, pH 2.0) with a flow-rate of 1.5 ml/min. HPLC was performed in isocratic mode for 20 min.

Vindoline and catharanthine were extracted following protocol of Lourdes Miranda-Ham et al. (2007). Briefly, oven dried young leaves (50 mg) collected from 2-month-old plants for vinblastine and roots (200 mg) from 1-year-old plants maintained in glass house for ajmalicine were extracted with 1 ml methanol by sonication (30 min). Samples were centrifuged at 12,000 rpm for 10 min at room temperature and the supernatant was decolorized (for leaf samples) by adding activated charcoal. The resulting methanolic extract was evaporated to dryness, resuspended in 20 µl methanol and used for HPLC quantification. The mobile phase consisted of a mixture of 5 mM Na2HPO4 (pH 6.0) (solvent A) and gradient grade methanol (solvent B) at a flow rate of 1.5 ml per min. The change in mobile phase was set to a linear gradient from 86:14 to 14:86 at 0–26 min in isocratic mode (14:86 v/v at 26–30 min), a linear gradient from 14:86 to 86:14 at 30–35 min, an isocratic elution with 14:86 (v/v) at 35–40 min. Data was extracted at 238 nm for secolloganin, 254 nm for vindoline, catharanthine, ajmalicine, and vinblastine. Peak area obtained from authentic standards (Sigma-Aldrich, St. Louis, MO, United States) (Supplementary Figure S2) and samples were used to quantify alkaloids and expressed as relative content in %.

Chlorophyll and Carotenoids Quantification, and Phenotypic Analyses
The amount of total chlorophyll and carotenoids was quantified according to Lichtenthaler (1987). Briefly, 20 mg of leaf (third developmental stage) was extracted in 98% ethanol for 2 h. The supernatant was used for quantification of chlorophyll and carotenoids. The absorbance was measured at 470, 653, and 666 nm and the amount of chlorophyll a, chlorophyll b, and total carotenoids was calculated. Flowers of T0 transgenic lines were selfed individually and siliques were collected separately from individual transgenic lines. The seeds were germinated and screened by PCR to select the first generation T1 positive plants from each transgenic line. Phenotypic parameters such as number of leaves, branches, flowers and siliques formed in WT and selected T1 transgenic lines were analyzed.

Copy Number Determination in Transgenic C. roseus Lines
Copy number of G(G)PPS and GES in transgenic G(G)PPS and G(G)PPS+GES lines were confirmed by using qPCR as described previously (Weng et al., 2004; Yi et al., 2008; Li et al., 2017). For absolute quantification, standard curve was generated using twofold dilutions of genomic DNA extracted from WT controls, selected G(G)PPS and all three lines of G(G)PPS+GES using primers of G(G)PPS, GES, and tryptophan decarboxylase [TDC, the endogenous single copy gene as determined by Godijn et al. (1994)]. Three separate sets of reactions were performed to amplify G(G)PPS, GES, and TDC. Each reaction (5 µl) contained
2.5 µl of 2X Maxima SYBR Green PCR master mix (Thermo Scientific, United States), 100 ng genomic DNA and 2 µM gene-specific primers. Reaction was performed as follows: 94°C for 10 min for first cycle, followed by 94°C for 15 s and 60°C for 15 s for 40 cycles. The PCR efficiency of target genes and TDC were calculated from slope of standard curve for each gene. The number of copies of G(G)PPS and GES were determined according to Pfaffl (2001).

### Statistical Analysis
Average mean, standard error (SE) and number of replicates \((n)\) used for individual experiment were employed for statistical analysis using the GraphPad QUICKCALC online software. Statistical significance of differences between control and samples were tested according to the unpaired Student’s \(t\)-test.

### RESULTS

#### Transient Overexpression of G(G)PPS and G(G)PPS+GES Resulted in Increased Alkaloid Accumulation

Transient overexpression by agroinfiltration either in the host plant or in heterologous systems has been widely used for determining the gene function. Moreover, it is an efficient strategy for functional analyses of genes in those plants where the study of gene function is limited owing to very low transformation efficiency and recalcitrant nature of the plant for genetic transformation. Hence, before proceeding for genetic transformation of \(C.\) roseus with G(G)PPS and G(G)PPS+GES, we carried out transient overexpression of these genes to determine their effect on MIA biosynthesis. As G(G)PPS and GES were previously reported to positively influence MIA biosynthesis in \(C.\) roseus (Rai et al., 2013; Kumar et al., 2015), we hypothesized that improved availability of precursors (GPP and geraniol) by overexpression of G(G)PPS and co-expression of G(G)PPS+GES could enhance MIA accumulation in \(C.\) roseus leaves. As the MIA content varies with leaf developmental stages, both genes were transiently overexpressed in first pair of leaves (that exhibit highest alkaloid accumulation). While, overexpression of G(G)PPS resulted in >30-fold transcript increase, co-expression of G(G)PPS+GES lead to ∼14- and 8-fold increase in transcripts of G(G)PPS and GES, respectively (Figures 2A,B). Next, to determine the effect of G(G)PPS and G(G)PPS+GES overexpression on alkaloid accumulation, first we checked the level of terpenoid intermediate secologanin. HPLC analysis showed ∼2.8- and 2.2-fold increase in secologanin levels in G(G)PPS and G(G)PPS+GES infiltrated leaves, respectively, compared to empty vector Pb1121 controls (Figures 2C,D and Supplementary Figures S3A,B). Subsequent analysis of the monomeric alkaloids in G(G)PPS infiltrated leaves revealed ∼3- and ∼6-fold increase in vindoline and catharanthine, respectively (Figure 2C and Supplementary Figure S3A). Similarly, G(G)PPS+GES co-infiltrated leaves exhibited an increase of ∼2.5 and ∼3.2-fold vindoline and catharanthine (Figure 2D and Supplementary Figure S3B). Higher accumulation of secologanin, vindoline and catharanthine could be attributed to dramatic increase in the availability of accumulated transcripts as a result of transient overexpression. Taken together, our results clearly demonstrated that overexpression of G(G)PPS and G(G)PPS+GES can improve the metabolic flux toward MIA biosynthesis in \(C.\) roseus.

#### Generation of Transgenic C. roseus Plants Overexpressing G(G)PPS and G(G)PPS+GES

In order to substantiate our findings of transient overexpression studies and to demonstrate the \(in\ planta\) role of G(G)PPS and G(G)PPS+GES in improving MIA production, we generated transgenic \(C.\) roseus overexpressing G(G)PPS and G(G)PPS+GES. So far, very few efforts have been made with respect to metabolic engineering of \(C.\) roseus plant due to its highly recalcitrant nature for genetic transformation together with low transformation efficiency. Lately, some reports have shown the generation of transgenic \(C.\) roseus through \(A.\) tumefaciens-mediated transformation. For the generation of transgenic \(C.\) roseus plants, culture conditions were optimized for callus induction and indirect regeneration following Wang Q. et al. (2012). In vitro grown hypocotyls were used as explants for Agrobacterium-mediated genetic transformation. Hypocotyl explants were precultured in MSCP1 (Figure 3Ai) for 3 days prior to Agrobacterium infection as it has been reported to improve the rate of transformation in \(C.\) roseus (Alam et al., 2017). After co-cultivation with Agrobacterium-harboring overexpression constructs, callus initiation was observed in most of the explants in a week in MSCP1 supplemented with 40 mg/L kanamycin and 300 mg/L carbenicillin. The cream colored friable calli developed after about 3 weeks were sub-cultured on to MSCP2 selection medium (Figure 3Aii). However, the calli upon subculturing in MSCP2 medium turned brown in most of the cases and died in about 1–2 weeks. After 2 weeks of sub-culturing in MSCP2 selection medium, only 2–3% of survived calli turned into green friable calli and shoot initiation was observed (Figure 3Aiii). Further, the initiated shoots were transferred to MSCP3 regeneration medium containing appropriate antibiotics. Although the regeneration efficiency was very less, multiple shoots were induced from embryogenic calli in some of the transformation events (Figure 3Aiv). From each calli, 3–4 shoots were induced and after 1 month of shoot maturation, healthy shoots were transferred initially to half strength MS medium supplemented with 2 mg/L IBA. After 2 weeks, though shoots exhibited root initiation, leaves turned yellow and exhibited necrotic symptoms in most of the cases. Hence, a lower concentration of IBA (2.4 µM) was used for root proliferation according to Dhandapani et al. (2008) (Figure 3Av). Plants with well developed roots were transferred to 1:1 sterile soilrite:vermicompost mix and maintained in glass house conditions (Figure 3Aii and Supplementary Figures S4A,B). The complete process of
transformation using hypocotyl explants from seed germination to hardening of putative transgenic lines took approximately 4 months. Regeneration efficiency from hypocotyl explants was very low as mentioned above and we were able to generate seven *C. roseus* lines overexpressing *G(G)PPS*. For PCR screening of putative transformants, 35S forward and two different *G(G)PPS* specific reverse primers were used. In both primer combinations, seven plants out of 18 kanamycin-selected plants were positive for *G(G)PPS* confirming the transgenic nature (Supplementary Figure S5A).

The regeneration efficiency from embryogenic calli was found to be low (3%) and there was no regeneration in the case of explants co-transformed with *G(G)PPS*+GES. Hence, an alternate approach of genetic transformation using nodal explants was followed for co-transformation. After co-cultivation with *Agrobacteria* harboring *G(G)PPS* and GES overexpression constructs, nodal explants were selected in MS-BNT medium supplemented with 70 mg/L kanamycin and 300 mg/L carbenicillin (Figure 3Bi). Most of the co-cultivated explants turned brown and died upon transferring to selection media. Healthy explants were sub-cultured for five more rounds in kanamycin selection media with 70 mg/L. Subsequently, the kanamycin concentration was increased to 100 mg/L for next two rounds. The explants exhibited initiation of leaves and shoot elongation from third round of selection (∼1 month after co-cultivation). In most of the survived explants after five rounds of antibiotic selection, shoot bud induction as well as proliferation occurred from nodes resulting in 3–7 multiple shoots (Figure 3Bii). Individual shoots were then transferred to fresh selection media every 15 days and healthy surviving shoots were inoculated into rooting medium. Although root initiation was observed in rooting
FIGURE 3 | Generation of transgenic C. roseus plants overexpressing G(G)PPS and GES. (A) Transformation of C. roseus using hypocotyl explants for G(G)PPS overexpression. (i) Preculture of hypocotyl explants in MS basal media supplemented with 250 mg/L proline and 150 mg/L casein hydrolysate (MSCP) media; (ii) callus induction in MSCP1 media supplemented with 40 mg/L kanamycin and 300 mg/L carbenicillin; (iii) callus proliferation and shoot initiation in MSCP2 with 70 mg/L kanamycin and 300 mg/L carbenicillin; (iv) shoot elongation in MSCP3 with 90 mg/L kanamycin and 300 mg/L carbenicillin; (v) root proliferation in half strength MS media with 2.4 µM IBA; (vi) Established transgenic G(G)PPS plant in the flowering stage. (B) Transformation of C. roseus nodal explants for G(G)PPS+GES overexpression. (i and ii) Selection of nodal explants and multiple shoot induction in MS media supplemented with 1 mg/L BAP + 0.1 mg/L NAA + 400 mg/L thiamine HCl + 70 or 100 mg/L kanamycin + 300 mg/L carbenicillin, respectively; (iii) root proliferation in half strength MS media with 2.4 µM IBA; (iv) Established transgenic G(G)PPS+GES plant in the flowering stage.

media containing 2 mg/L IBA, root proliferation was only obtained in a much lower IBA (2.4 µM) concentration (Figure 3Bii). Well rooted plants were transferred to pots containing 1:1 sterile soil:vermicompost mix (Figure 3Biv and Supplementary Figures S4A,B). After obtaining putative transformants of G(G)PPS+GES, PCR analysis using genomic DNA isolated from leaves was carried out to identify transgenic nature of plants. Screening of G(G)PPS+GES lines was carried out by using nptII forward and reverse primers, and 35S forward and gene specific [(G(G)PPS or GES)] reverse primers. PCR results indicated three plants positive for G(G)PPS+GES out of 16 kanamycin selected plants (Supplementary Figures S5B,C). The transformation efficiency using nodal explants was also ~3% similar to transformation using hypocotyl explants. PCR analysis using genomic DNA with primers specific for Agrobacterium chromosomal virulence gene (ChvA) revealed the absence of the corresponding band, thus ruling out the possibility of Agrobacterium contamination in both G(G)PPS and G(G)PPS+GES transformants (Supplementary Figure S6).

Ectopic Expression of G(G)PPS and G(G)PPS+GES Elevates Alkaloid Accumulation in C. roseus Transgenic Plants

Having confirmed the transgenic nature of C. roseus plants, the transcript abundance of G(G)PPS and GES in transgenic lines was analyzed by qRT-PCR. First, the transcript levels in all transgenic lines were determined with respect to WT control plants that were also generated through tissue culture. The various transgenic lines showed increased expression of G(G)PPS with a 2- to 9.5-fold increase compared to WT control plants, with maximum expression in G(G)PPS_5 followed by 8-fold in G(G)PPS_3 and G(G)PPS_7 (Figure 4A). Transgenic plants co-expressing G(G)PPS+GES showed increased expression of
both genes (Figure 4B). The expression of \(G(G)PPS\) was \(\sim 7\), \(3\), and \(4\)-fold in lines 1, 2 and 3, respectively, whereas the expression of \(GES\) was \(\sim 5\)-fold in line 1, and \(\sim 3\)-fold in lines 2 and 3 (Figure 4B). Next, to determine whether the changes in gene expression were also accompanied by increased MIA accumulation, metabolites were extracted from independent transgenic lines and quantified by HPLC. The content of terpenoid intermediate secologanin exhibited a significant boost of \(\sim 2\)-fold in lines \(G(G)PPS_3\), \(G(G)PPS_5\) and \(G(G)PPS_7\) that also showed highest transcript abundance (Figure 5A and Supplementary Figure S7A). There was no significant enhancement of secologanin in the remaining four \(G(G)PPS\) lines that correlated with the gene expression of \(G(G)PPS\) (Figure 5A). Further, to check whether improved availability of secologanin resulted in enhanced accumulation of monomeric alkaloids in leaves, vindoline and catharanthine levels were analyzed in \(G(G)PPS\) transgenic lines. HPLC analysis revealed that the trend in accumulation of monomers correlated with the level of secologanin in all seven lines with significant increase of 1.5- to 1.7-fold vindoline and 1.7- to 2.0-fold catharanthine in lines \(G(G)PPS_3\), \(G(G)PPS_5\) and \(G(G)PPS_7\) (Figure 5A and Supplementary Figure S7A). Similarly, \(G(G)PPS+GES\) co-expressing lines exhibited a significant increase in secologanin (\(\sim 1.8\)-fold) along with significant increase in vindoline (1.6- to 2.2-fold) and catharanthine (2.5- to 3.0-fold) (Figure 5B and Supplementary Figure S7B). Although all three \(G(G)PPS+GES\) lines showed significant accumulation of secologanin and vindoline similar to \(G(G)PPS\) lines, the level of catharanthine was
FIGURE 5 | Quantification of metabolites in the T<sub>0</sub> lines of transgenic G(G)PPS and GES <i>C. roseus</i> plants. Relative amounts of secologanin, vindoline and catharanthine (A,B), ajmalicine (C,D), and vinblastine (E,F) in transgenic G(G)PPS and G(G)PPS+GES <i>C. roseus</i> lines. Secologanin was extracted from 50 mg (fresh weight) of leaves and quantified following Tikhomiroff and Jolicoeur (2002). The monomeric alkaloids vindoline and catharanthine were extracted using 10 mg of oven dried leaves according to Lourdes Miranda-Ham et al. (2007), and quantified following Kumar et al. (2015). Ajmalicine was extracted from roots of wild type (WT) and transgenic plants using 200 mg fresh weight tissue following Pan et al. (2016). The methanolic extract was subjected to HPLC analysis to quantify ajmalicine. The dimeric alkaloid vinblastine was extracted using 50 mg of oven dried leaves and quantified according to Pan et al. (2016). In all cases, young leaves or roots of same developmental stages were used for alkaloid extraction and were quantified by using HPLC. The quantified metabolites are expressed as % levels relative to WT controls. Error bars represent mean ± standard error (SE) of three to four independent experiments. Significant differences at <i>P</i> < 0.05, <i>P</i> < 0.01, and <i>P</i> < 0.001 are represented by "∗", "∗∗", and "∗∗∗", respectively.

much higher (>3.0-fold) in co-expressing lines (Figure 5B and Supplementary Figure S7B).

In MIA biosynthesis, 4,21-dehydrogeissoschizine acts as a branch point intermediate for the formation of ajmalicine and stemmadenine. While ajmalicine is converted to serpentine, stemmadenine undergoes several transformations to form catharanthine and vindoline (Peebles et al., 2011). In order to assess the impact of G(G)PPS and GES overexpression on accumulation of root alkaloid, ajmalicine was quantified in roots of WT controls and transgenic lines by HPLC analysis. Consistent with the effect on secologanin, catharanthine and vindoline levels, G(G)PPS and GES overexpression enhanced the level of ajmalicine by ~1.25- to 1.5-fold in three high expressing G(G)PPS transgenic lines (Figure 5C) and 1.3- to 1.8-fold in G(G)PPS+GES co-expressing lines (Figure 5D). This indicated that improved availability of GPP and geraniol due
to overexpression of \(G(G)PPS\) and GES also influenced the accumulation of alkaloids in roots.

### Differential Expression of Peroxidise 1 in Transgenic Lines Correlated With Vinblastine Accumulation

A significant increase of vinblastine (~1.5-fold) was observed in three \(G(G)PPS\) overexpressing lines that exhibited increased secologanin, vindoline and catharanthine (Figures 5A,E). With respect to alkaloid accumulation in \(G(G)PPS+GES\) co-expressing plants, although there was elevated accumulation of terpenoid intermediate secologanin, and monomeric alkaloids, there was no significant increase in the level of vinblastine in any of the lines (Figures 5B,F). In a similar manner, when ORCA3 was expressed alone or co-expressed with \(G10H\) in transgenic \(C. roseus\) plants, monomers were significantly enhanced without any effect on accumulation of \(\alpha-3',4'-anhydrovinblastine\) and vinblastine (Pan et al., 2012). Peroxidise 1 (PRX1) mediates the coupling of monomeric catharanthine and vindoline into dimeric \(\alpha-3',4'-\)anhydrovinblastine, the immediate precursor for vinblastine (Costa et al., 2008). The fact that, the vinblastine level was boosted only in \(G(G)PPS\) transgenic plants and not in \(G(G)PPS+GES\) lines indicated a possible differential regulation at the terminal step involving PRX1. To verify this, transcript levels of PRX1 were determined in \(G(G)PPS\) and \(G(G)PPS+GES\) transgenic \(C. roseus\) lines. The analyses revealed a significant difference of PRX1 transcripts levels in \(G(G)PPS\) and \(G(G)PPS+GES\) transgenic lines (Figure 6). All three higher alkaloid accumulating \(G(G)PPS\) lines \([G(G)PPS_3, G(G)PPS_5, \text{and} G(G)PPS_7]\) showed enhanced PRX1 transcript abundance ranging from fourfold to eightfold (Figure 6A). However, \(G(G)PPS+GES\) co-expressing lines exhibited PRX1 expression similar to that of WT controls (Figure 6B). This differential expression of PRX1 could be the possible reason for contrasting levels of vinblastine in \(G(G)PPS\) and \(G(G)PPS+GES\) transgenic plants.

### Transgenic \(C. roseus\) Plants Exhibited Stable Levels of Gene Expression and Alkaloids in the T1 Generation

To determine the genetic and chemostability, we checked the transcript and metabolite levels in PCR positive T1 lines of \(G(G)PPS\) and \(G(G)PPS+GES\) (Figure 7 and Supplementary Figures S8, S9). First, transgene copy numbers in selected \(G(G)PPS\) \([G(G)PPS_1, G(G)PPS_3, G(G)PPS_5, \text{and} G(G)PPS_7]\) and \(G(G)PPS+GES\) transgenic lines were determined by qPCR using genomic DNA extracted from WT controls and transgenic lines. The correlation coefficients of the standard curves of target genes, \(G(G)PPS\), GES, and TDC internal control were 0.996–0.998, and PCR efficiencies were close to 97–100%. This indicated the accuracy and robustness in estimating copy number of genes based on the standard curves. The analysis revealed that \(G(G)PPS_1\), \(G(G)PPS_3\), and \(G(G)PPS_7\) showed the presence of two copies, whereas line \(G(G)PPS_5\) exhibited the presence of three copies of \(G(G)PPS\) (Supplementary Table S2). With respect to co-expressing lines, \(G(G)PPS+GES_1\) showed three copies, whereas lines \(G(G)PPS+GES_2\) and \(G(G)PPS+GES_3\) possessed two copies of \(G(G)PPS\). The copy number of GES was found to be three in all the co-expressing transgenic lines (Supplementary Table S2). The WT controls showed one copy each for endogenous \(G(G)PPS\) and GES. Hence, the determined copy numbers also include the endogenous copy of \(G(G)PPS\) and GES in all transgenic events.

Transcript levels in the T1 \(G(G)PPS\) transgenic plants exhibited higher \(G(G)PPS\) expression (8- to 10-fold) in \(G(G)PPS_5\) and \(G(G)PPS_7\) lines (Supplementary Figure S9A) similar to the levels observed in the T0 lines. However, \(G(G)PPS_3\) line that showed significantly higher transcript accumulation (~8-fold) in the T0 generation, exhibited reduced expression in the T1 generation was comparable to WT control plants (Figure 4A and Supplementary Figure S9A). In co-transformed \(C. roseus\) lines, \(G(G)PPS\) transcript abundance was sixfold in line \(G(G)PPS+GES_1\) and ~3-fold in other two lines (Supplementary Figure S9B), whereas the
expression of GES varied between threefold and fourfold in all three lines (Supplementary Figure S9B). To confirm whether increased gene expression was accompanied by elevated MIA accumulation in transgenic lines, alkaloids were quantified in T1 G(G)PPS and G(G)PPS+GES lines. Similar to the accumulation observed in the T0 generation, T1 G(G)PPS lines exhibited improved accumulation of monomeric alkaloids with no significant change in levels of secologanin (Figure 7A and Supplementary Figure S10A). While G(G)PPS_5 and G(G)PPS_7 exhibited ~2.5-to 2.7-fold accumulation of vindoline and catharanthine, G(G)PPS_3 showed significant increase of only vindoline (~1.8-fold) (Figure 7A). As for the dimeric alkaloids, only G(G)PPS_5 and G(G)PPS_7 exhibited ~1.5-fold increase in vinblastine compared to WT controls (Figure 7C and Supplementary Figure S10A). Among the co-expressing G(G)PPS+GES plants, only two T1 plants showed improved accumulation of secologanin, (~1.5-fold), vindoline (~2.2-fold), and catharanthine (~2.8-fold) (Figure 7B and Supplementary Figure S10B). Also, similar to the T0 plants, there was no increase in the level of vinblastine in any of the T1 G(G)PPS+GES co-expressing lines possibly due to regulation of PRX1 (Figure 7D and Supplementary Figure S10B).

**DISCUSSION**

Vinblastine and vincristine are blockbuster anti-cancer drugs extracted exclusively from C. roseus leaves. As these dimeric MIAs are accumulated at very low levels in leaves, increasing their foliar concentrations would reduce the cost of production. Hence, this study was taken up to enhance the production of monomeric and dimeric MIAs by overexpression of early secoiridoid pathway genes in transgenic C. roseus plants. Previous efforts of overexpression or silencing of transcriptional regulators and pathway genes in C. roseus cell cultures have had limited success in improving MIAs production, indicating a complex regulatory mechanism that balances metabolic flux and thus MIAs accumulation in C. roseus (Murata and De Luca, 2005; Peebles et al., 2009; Jaggi et al., 2011; Van Moerkercke et al., 2015; Sun et al., 2017). In order to allow higher metabolic outputs, improving the availability of upstream precursors is an alternative strategy. Initial elicitation and feeding studies using indole and terpenoid precursors in C. roseus cell cultures indicated inconsistent results on accumulation of different MIAs including serpentine, ajmalicine, tabersonine, and strictosidine (Moreno et al., 1993; Whitmer et al., 1998;
Contin et al., 1999). However, later studies in cell suspension level that GPPS and GES play important roles in providing the flux toward downstream MIA biosynthesis in _C. roseus_ (Rai et al., 2013; Kumar et al., 2015). Since GPPS and GES provide starting precursors (GPP and geraniol) for the monomeric MIAs (Figure 2 and Supplementary Figure S3), indicating that stable overexpression of these genes in transgenic _C. roseus_ plants could result in improved production of MIAs.

The lack of a reliable genetic transformation method in _C. roseus_ at the whole plant level has been a major impediment for improving MIAs by metabolic engineering approaches (Zarate and Verpoorte, 2007). Owing to the recalcitrant nature and low transformation efficiency, only a few reports have demonstrated genetic transformation in _C. roseus_ using genes related to MIA biosynthesis (Pan et al., 2012; Wang Q. et al., 2012). Here, we generated transgenic _C. roseus_ overexpressing _G(G)PPS_ using transformation protocol of Wang Q. et al. (2012) with minor modifications. Although Wang Q. et al. (2012) reported transformation efficiency of 11% for transgenic _C. roseus_ expressing _DAT_ gene, in our case the efficiency of transformation was ∼3%. As hypocotyl explants co-cultivated with _G(G)PPS_-GES constructs failed to generate shoots, an alternate regeneration method using nodal explants as reported by Verma and Mathur (2011a) was employed for transformation. This strategy yielded only three transformants with ∼3% transformation efficiency. The lower transformation efficiency in this study could be due to the genotype of _C. roseus_ used as it has been shown in other plant species that transformation efficiency depends on the genotype and cultivar (Wang Y. et al., 2012; Udayakumar et al., 2014). Transgenic _G(G)PPS_ and _G(G)PPS+GES_ lines exhibited varied levels of transcript abundance for corresponding genes (Figure 4). _G(G)PPS_ expression ranged from 2- to 9.5-fold and 3- to 6-fold in _G(G)PPS_ and _G(G)PPS+GES_ transgenic lines, respectively, whereas _GES_ expression was 3- to 4.5-fold in co-expressing lines (Figure 4). Similar to our observation, overexpression of _DAT_ in transgenic _C. roseus_ resulted in twofold to sevenfold expression in different lines (Wang Q. et al., 2012). In another report, overexpression of transcriptional regulator _ORCA3_ alone in transgenic _C. roseus_ plants resulted only in ∼2-fold expression, whereas transgenic lines co-expressing _G10H_ and _ORCA3_ resulted in ∼5- and 7-fold increase in transcript levels of _G10H_ and _ORCA3_ respectively (Pan et al., 2012). Increased transcript accumulation of _G(G)PPS_ and _G(G)PPS+GES_ in transgenic _C. roseus_ plants enhanced the content of secoiridoid secolagani, monomeric vindoline and catharanthine, and root alkaloid ajmalicine (Figures 5 and Supplementary Figure S7). Overall, the level of secolagani and vindoline in three higher expressing _G(G)PPS_ lines and in all _G(G)PPS+GES_ transgenic lines remained similar (Figures 5A,B). However, there was higher accumulation of catharanthine in two _G(G)PPS+GES_ lines compared to _G(G)PPS_ lines (Figures 5A,B), suggesting that improved availability of both GPP and geraniol could have enhanced the accumulation of catharanthine. It was reported that co-expression of 1-deoxy-d-xylulose-5-phosphate synthase (DXS) and _G10H_ in hairy roots resulted in improved accumulation of ajmalicine and tabersonine compared to expression of DXS alone (Peebles et al., 2011). Similarly, _C. roseus_ transgenic plants co-expressing _ORCA3_ and _G10H_ exhibited higher production of ajmalicine compared to plants expressing _ORCA3_ alone (Pan et al., 2012). These prior findings along with our present results suggest that overexpression of multiple genes may be necessary to achieve significant gains in product accumulation especially in pathways involving multiple branches, where precursors are channeled into diverse metabolites (Peebles et al., 2011).

In addition to enhancing secolagani and monomeric alkaloids, _G(G)PPS_ overexpressing lines also accumulated higher level of vinblastine (Figure 5E and Supplementary Figure S7A). In contrast, _G(G)PPS+GES_ lines did not show any increase in the levels of vinblastine despite of significant increase in secolagani, vindoline, and catharanthine (Figure 5F). This result suggested that improved metabolic flux (in the form of monomeric alkaloids) is somehow not being utilized for dimeric alkaloid formation. These findings prompted us to check whether gene expression of _PRX1_ has any influence on the accumulation of vinblastine. Transcript analysis revealed that _G(G)PPS_ and _G(G)PPS+GES_ overexpressors had a differential expression of _PRX1_ that correlated with the accumulation of vinblastine (Figures 5E,F and Supplementary Figure S7). It is not clear at this point, how _PRX1_ is differentially expressed in _G(G)PPS_ and _G(G)PPS+GES_ overexpressors. However, _PRX1_ may be transcriptionally regulated by some unknown mechanism in _G(G)PPS_ overexpressors leading to enhanced vinblastine. It has been previously reported that the expression of _PRX1_ and accumulation of 3′,4′-anhydrovinblastine is under complex regulation and also depends on the availability of hydrogen peroxide (Costa et al., 2008). In addition, _PRX1_ has been suggested to act as positive regulator and interacts with ethylene responsive factor (ERF) regulating accumulation of 3′,4′-anhydrovinblastine in _C. roseus_ (Wang et al., 2016).

The success of genetic engineering depends on genetic and chemical stability of transgenic plants in subsequent generations (Verma et al., 2015). The copy number of integrated gene affects the expression level and genetic stability in transgenic
Overexpression of *GPPS* and some of the terpene synthases redirects metabolic flux toward secondary metabolite biosynthesis, thus lowering the flux for the synthesis of other primary metabolites thereby affecting plant growth. For instance, transgenic tobacco plants expressing snapdragon *GPS.SSU* were dwarf and displayed strong chlorosis with reduced chlorophyll content (Orlova et al., 2009). Also, overexpression of strawberry linalool/nerolidol synthase and taxadiene synthase in *Arabidopsis* resulted in a dwarf phenotype due to reduction in gibberellic acid levels (Aharoni et al., 2003; Besumibes et al., 2004). In contrast, co-expression of peppermint *GPPS.SSU* with different monoterpene synthases enhanced the monoterpene production without affecting plant growth in tobacco (Yin et al., 2017). Here, overexpression of *G(G)PPS* alone or together with *GES* did not alter the *C. roseus* plant phenotype (Supplementary Table S3 and Supplementary Figure S4C). Selected transgenic lines were tested for growth parameters and no differences were found (Supplementary Table S3). As we have overexpressed a bifunctional *G(G)PPS*, it could have provided higher flux of GGPP and GPP, respectively, required for generalized and specialized metabolism in transgenic lines, thereby not affecting the plant growth. Indeed, two best expressing *G(G)PPS* transgenic lines exhibited a significant increase in the level of total chlorophyll indicating that enhanced GGPP production by the bifunctional enzyme could have resulted in improved chlorophyll content (Supplementary Figure S11). Although overexpression of *Picea abies* bifunctional *G(G)PPS* resulted in increased accumulation of geranylgeranyl fatty acid esters, chlorophylls and carotenoids remained unaffected (Nagel et al., 2014).

In summary, we demonstrate that the level of monomeric MIAs and pharmaceutically important vinblastine can be enhanced in plants by transgenic overexpression of genes involved in early steps of secoiridoid pathway. While overexpression of bifunctional *G(G)PPS* enhanced both monomeric and dimeric MIAs, co-expression of *G(G)PPS* and *GES* led to increase of monomeric MIAs higher than *G(G)PPS* overexpressors. Improved accumulation of MIAs in transgenic periwinkle without compromising the plant growth indicated the positive role of bifunctional *G(G)PPS* in both generalized and specialized metabolism. Commercial exploitation of *G(G)PPS* transgenic lines could reduce the cost of dimeric alkaloids production and co-expressing lines could be exploited for vindoline and catharanthine production or may be used in molecular breeding approaches to further improve the dimeric alkaloids content. Moreover, future research focusing on stacking of a combination of MIA pathway genes including *PRX1* and transcriptional regulators could enhance the flux resulting in further improvement of alkaloids in *C. roseus*.

**AUTHOR CONTRIBUTIONS**

SK and HS performed the experiments. SK, HS, and DN analyzed the data. DN conceived and coordinated the research. SK and DN wrote the manuscript.

**FUNDING**

This work was supported by Department of Biotechnology, Government of India funded project (BT/PR6109/AGII/106/857/2012; GAP-272) to DN. SK was initially supported by Research Associateship by GAP-272 and later by CSIR Senior Research Associateship. HS acknowledges ICMR for the Research Fellowship.

**ACKNOWLEDGMENTS**

The authors express their sincere gratitude to the Director, CSIR-CIMAP for his support throughout the study. Authors are also thankful to Dr. Micheal Long for his help with English corrections. Institutional communication number for this article is CIMAP/PUB/2018/01.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00942/full#supplementary-material
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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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