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REVIEW ARTICLE

Emerging trends in research on spatial and temporal organization of terpenoid indole alkaloid pathway in *Catharanthus roseus*: a literature update

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Abstract *Catharanthus roseus* (The Madagaskar Periwinkle) plant is commercially valued for harbouring more than 130 bioactive terpenoid indole alkaloids (TIAs). Amongst these, two of the leaf-derived bisindole alkaloids—vinblastine and vincristine—are widely used in several anticancer chemotherapies. The great pharmacological values, low *in planta* occurrence, unavailability of synthetic substitutes and exorbitant market cost of these alkaloids have prompted scientists to understand the basic architecture and regulation of biosynthesis of these TIAs in *C. roseus* plant and its cultured tissues. The knowledge gathered over a period of 30 years suggests that the TIA biosynthesis is highly regulated by developmental and environmental factors and operates through a complex multi-step enzymatic network. Extensive spatial and temporal cross talking also occurs at inter- and intracellular levels in different plant organs during TIA biogenesis. A close association of indole, methylerythritol phosphate and secoiridoid monoterpenoid pathways and involvement of at least four cell types (epidermis, internal phloem-associated parenchyma, laticifers and idioblasts) and five intracellular compartments (chloroplast, vacuole, nucleus, endoplasmic reticulum and cytosol) have been implicated with this biosynthetic mechanism. Accordingly, the research in this area is primarily advancing today to address and resolve six major issues namely: precise localization and expression of pathway enzymes using modern in situ RNA hybridization tools, mechanisms of intra- and intercellular trafficking of pathway intermediates, cloning and functional validation of genes coding for known or hitherto unknown pathway enzymes, mechanism of global regulation of the pathway by transcription factors, control of relative diversion of metabolite flux at crucial branch points and finally, strategising the metabolic engineering approaches to improve the productivity of the desired TIAs in plant or corresponding cultured tissues. The present literature update has been compiled to provide a brief overview of some of the emerging developments in our current understanding of TIA metabolism in *C. roseus*.

Keywords *Catharanthus roseus* · Terpenoid indole alkaloids · Pathway architecture · Spatial and temporal organization

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Introduction

Terpenoid indole alkaloids (TIAs), elegantly represented in *Catharanthus roseus*, are a large group of about 2,000 naturally occurring compounds widely distributed amongst the members of the family Apocynaceae, Loganiaceae and Rubiaceae. Majority of these compounds have been implicated with a role in plant defence mechanisms against pests and pathogen (Luijendijk et al. 1996; Roepke et al. 2010; Guirimand et al. 2010b). The complex chemistry of several TIAs has also been corroborated with their strong biological activities against a variety of human health

disorders (van der Heijden et al. 2004). Molecules like CNS suppressant reserpine, antihypertensive serpentine, vasodilatory yohimbine, antimalarial quinine, antiarrythmic ajmalicine and most importantly the anticancer vinblastine and vincristine that are being widely used as prescription drugs in the pharma sector belong to this group of plant alkaloids. It is therefore not surprising that a wealth of information today exists regarding their biosynthesis at the level of enzymes and corresponding genes (Verpoorte et al. 1997; Shanks et al. 1998; Rischer et al. 2006; O'Connor and Maresh 2006). The knowledge gathered over the last three decades suggests that TIA synthesis in *C. roseus* is strictly regulated at the level of pathway genes and enzymes that are differentially expressed at discrete inter- and intracellular locations under the influence of several developmental and ecophysiological and environmental signals. These developments have been periodically reviewed in published literature (Kutchan 1995, 2005; Verpoorte et al. 1997; De Luca and St. Pierre 2000; Verpoorte and Alfermann 2000; Facchini 2001, 2006; O'Connor and Maresh 2006; Rischer et al. 2006; Facchini and De Luca 2008; Mahroug et al. 2007; Barleben et al. 2007; El-Sayed and Verpoorte 2007; Loyola-Vargas et al. 2007; Oudin et al. 2007a; Roytrakul and Verpoorte 2007; Stockigt and Panjikar 2007; Ziegler and Facchini 2008; Guirimand et al. 2010a).

From a holistic perspective, the biosynthesis of TIAs in *C. roseus* proceeds via 30 coordinately regulated enzymatic steps involving at least 35 known intermediates (van der Heijden et al. 2004; Facchini and De Luca 2008). Consequently, 30 biogenetic and 2 regulatory genes have also been identified for their close association with this pathway. Out of a total of 42 cDNA clones identified in major TIA-producing plants, 25 belong to *C. roseus* (Guirimand et al. 2010a). All TIAs are biosynthesised from a central precursor molecule—strictosidine—which is a condensation product of an indole ring donor tryptamine and a terpenoid moiety donor secologanin (Fig. 1). Strictosidine that heralds the first indication of a switchover of carbon flux from primary to secondary metabolism during TIA synthesis is subsequently reacted upon by the enzyme strictosidine β-glucosidase (SGD) to yield a highly reactive ring-opened dialdehyde. SGD is now known to induce different types of structural rearrangements in the resultant unstable aglycon to yield various types of TIA skeletons (Geerlings et al. 2000) and is likely to be the key factor for apparent tissue- as well as genotype-specific diversity of TIA spectrum in plants (Verpoorte et al. 1997). The unstable aglycon undergoes several unknown conversions to yield cathenamine, which is a branch point intermediate for the synthesis of two monomeric alkaloids, catharanthine and vindoline. While very little is known about the catharanthine subway (Loyola-Vargas et al. 2007), the vindoline route is fairly well dissected at the level of associated enzymes and genes (Scroder et al. 1999; St. Pierre and De Luca 1995; Vazquez-Flota et al. 1997; St. Pierre et al. 1998; Laflamme et al. 2001). Diversion of flux from cathenamine-derived intermediate tabersonine to vindoline is facilitated by six enzymatic steps in the aerial tissues of *C. roseus* plants. The biochemistry of this six-step route involves three hydroxylations and one each of *O*-methylation, *N*-methylation and *O*-acetylation reactions. The tabersonine to 16-hydroxytabersonine conversion is catalysed by a cytochrome P450-dependent tabersonine-16-hydroxylase (T16H) enzyme, followed by its methylation by 16-hydroxytabersonine-16-*O*-methyl transferase (16OMT) which also requires 5-adenosyl-L-methionine as a co-substrate (Levac et al. 2008). The conversion of 16-methoxy tabersonine to 16 methoxy-2, 3-dihydrotabersonine then occurs via an uncharacterised oxidation step. The subsequent step involves a thylakoid-associated *N*-methyltransferase (NMT) to obtain deacetoxyvindoline. The last two biogenetic reactions are catalysed by a light-regulated deacetoxyvindoline-4-hydroxylase (D4H) and deacetylvindoline-4-*O*-acetyltransferase (DAT) enzymes that are expressed only in special idioblast/laticifer cells in leaves (De Luca et al. 1986; De Carolis et al. 1990; St. Pierre et al. 1998; Campous-Tamayo et al. 2008; Shukla et al. 2010; Guirimand et al. 2011a). In contrast to aerial tissues, the roots of *C. roseus* plants operate another subway from tabersonine to lochnericine by the catalytic action of tabersonine 6, 7-epoxidase (T6,7E; Rodriguez et al. 2003). Lochnericine can then be converted into horhammericine by tabersonine 19-hydroxylase. Alternatively, tabersonine can also be routed towards horhammericine via 6,7 dehydrominovincine, and both minovincinine and/or horhammericine can then be acetylated to yield 19-*O*-acetylhorhammericine by the enzyme minovincinine 19-hydroxy-*O*-acetyl transferase (MAT) which is localised in cortical cells of growing root tips. Finally, the coupling of monomeric TIA catharanthine and vindoline resulting in the formation of vinblastine and vincristine in leaves marks the termination of TIA biogenetic pathway in *C. roseus*. Since vincristine and vinblastine are highly antimitotic molecules due to their inhibitory action on spindle assembly during cell division, this coupling reaction exclusively takes place in the cell vacuole as a measure of cellular containment. The dimerization reaction between catharanthine and vindoline is facilitated by class III basic peroxidase (Prx1; Sottomayor et al. 1996, 1998; Sottomayor and Ros Barcelo 2003; Kumar et al. 2007, 2011; Costa et al. 2008).

Intervention of an expanding tool box consisting of in situ RNA hybridization coupled with epi-fluorescence imaging, immunolocalization, X-ray crystallography, homology modelling, transcriptome profiling and ultra-

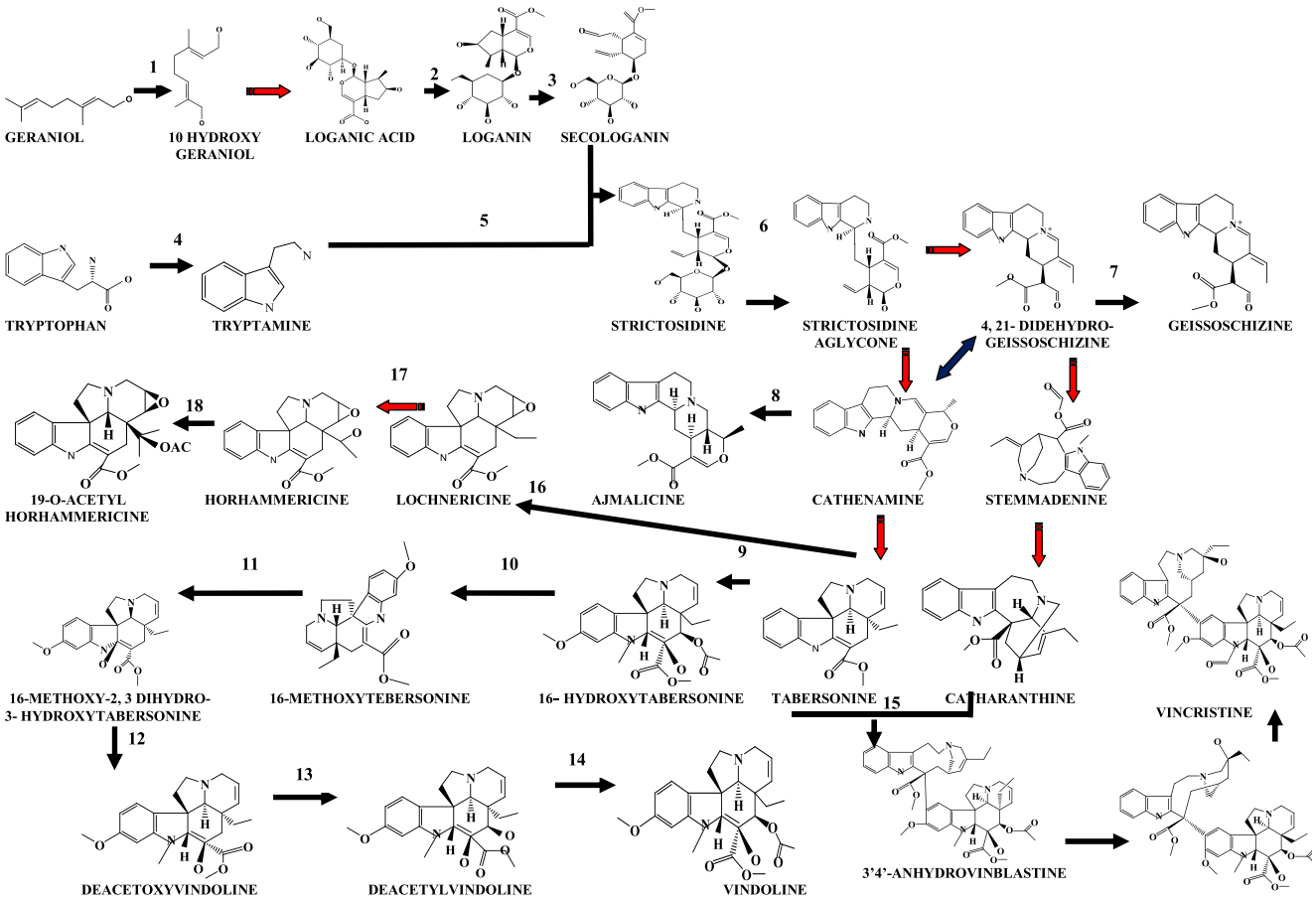

Fig. 1 The basic skeleton of terpenoid indole alkaloid biosynthesis in *C. roseus*. *1* Geraniol-10-hydroxylase (G10H), *2* loganic acid methyltransferase (LAMT), *3* secologanin synthase (SLS), *4* TDC, tryptophan decarboxylase, *5* strictosidine synthase (STR), *6* strictosidine β-glucosidase (SGD), *7* geissoschizine dehydrogenase (GDH), *8* cathenamine reductase (CR), *9* tabersonine 16-hydroxylase (T16H), *10* 16-hydroxytabersonine-16-*O*-methyltransferase (16OMT), *11* uncharacterized enzyme, *12* 16-methoxy-2,3-dihydro-3-hydroxy-tabersonine *N*-methyltransferase (NMT), *13* deacetoxyvindoline 4-hydroxylase (D4H), *14* 6-17-*O*-deacetylvindoline *O*-acetyltransferase (DAT), *15* class III basic peroxidase (PRX1), *16* tabersonine 6,7-epoxidase (T6,7E), *17* tabersonine 19 hydroxylase (T19H), *18* minovincinine-19-*O*-acetyltransferase (MAT)

sensitive metabolite quantification techniques in the last few years is continuously refining our understanding of TIA metabolism in *C. roseus*. Some of the fresh insights into the spatial and temporal organization/regulation of TIA biosynthesis are summarised in this literature update.

Basic architecture of enzymatic network during TIA biosynthesis in *C. roseus*—a resume

The TIA pathway begins via convergence to two primary metabolic routes, i.e. the shikimate and the secoiridoid pathways that respectively provide the indole and the terpene moieties to the basic TIA backbone (Fig. 1). The shikimate pathway leads to the formation of aromatic amino acid tryptophan which serves as the sole donor of indole ring to a variety of plant metabolites including auxins, glucosinolates, phytoalexins, TIAs, etc. (Herrmann 1995; Knaggs 2001; Arcuri et al. 2010). Anthranilate synthase (AS), which is a key regulatory enzyme associated with tryptophan synthesis from chorismate, is strongly feedback inhibited by tryptophan which binds to an allosteric site on the AS catalytic α-subunit (Radwanski and Last 1995). An altered form of α-subunit of AS which is less sensitive to tryptophan-mediated feedback inhibition has also been isolated from mutant cell lines resistant to tryptophan analogues like 5-methyl tryptophan in many plant systems including *C. roseus* as a possible strategy to avoid the stiff competition for tryptophan availability for TIA synthesis (Scott et al. 1979; Brotherton et al. 1996; Song et al. 1998; Seth and Mathur 2005). Tryptophan is decarboxylated to form tryptamine by the catalytic action of the enzyme tryptophan decarboxylase encoded by TDC gene (Noe and Berlin 1984; Goddijin et al. 1992). The TDC

gene is localised in cytosol (De Luca and Cutler 1987) and feedback inhibited by tryptamine (Meijer et al. 1993a, b). TDC transcription has been shown to be induced by several biotic and abiotic elicitors like jasmonates, salicylic acid, vanadyl sulphate and fungal homogenate (Pasquali et al. 1992; Rijhwani and Shanks 1998; Vazquez-Flota and De Luca 1998; Ouwerkerk and Memelink 1999) and, as such, represents an attractive target in metabolic engineering of TIA pathway (Canel et al. 1998; Guillet et al. 2000). However, the efforts made to overexpress TDC gene to boost TIA synthesis have not met with expected level of success (Merillon et al. 1986; Facchini and DiCosmo 1991; Meijer et al. 1993a, b; Moreno et al. 1995; Burlat et al. 2004) because TDC overexpression is probably dependent on sufficient tryptophan availability, rapid utilization of tryptamine and its subsequent translocations across tonoplast (Whitmer et al. 1998, 2003).

The biosynthesis of secologanin, that contributes the terpenoid moiety to TIA skeleton, proceeds via methylerythritol phosphate (MEP) pathway (Oudin et al. 2007a, b). The resultant isopentenyl diphosphate (IPP) and dimethylallyl diphosphate are condensed in a head-to-tail fashion in the presence of GPP synthase to yield geranyl diphosphate (GPP), which is a universal precursor for all monoterpenes including the secoiridoid secologanin (Contin et al. 1998; Hedhili et al. 2007). Though a general agreement regarding major involvement of MEP pathway in TIA synthesis in *C. roseus* exists today (Contin et al. 1998; Hong et al. 2003), the possibility of the cross talk between MEP and mevalonate routes for IPP synthesis cannot be completely ruled out (Ayora-Talavera et al. 2002). Such cross talking between MEP and mevalonic acid (MVA) pathways has also been proposed to occur during β-carotene, phytol and luteine synthesis in *C. roseus* (Arigoni et al. 1997). Sequencing of cDNA libraries of *C. roseus*, derived from leaf epidermome, has also indicated the presence of four MVA pathway genes [i.e. 3-hydroxy-3-methylglutaryl CoA reductase, 3-ketoacyl-CoA thiolase, acetoacetyl CoA thiolase and HMG CoA synthase] and three common genes of MEP and MVA pathways [i.e. isopentenyl pyrophosphate isomerase, farnesyl pyrophosphate synthase and geranyl pyrophosphate synthase] in the epidermis (Murata et al. 2006, 2008). The enzymology around multi-step conversion of GPP to secologanin via geraniol is still not very clear. Only four cDNAs encoding the P450-dependent geraniol 10-hydroxylase (G10H; Collu et al. 2001), acyclic monoterpene primary alcohol dehydrogenase (Ikeda et al. 1991), loganic acid methyltransferase (LAMT; Murata et al. 2008) and P450-dependent secologanin synthase (SLS; Irmler et al. 2000) have been identified. It has been proposed that at least 11 enzymatic steps may be occurring during geraniol to secologanin formation via loganic acid and loganin (Loyola-Vargas et al. 2007). The most important regulatory step in the synthesis of secoiridoid precursor of TIAs is the oxygenation of geraniol by G10H enzyme. This membrane-bound monooxygenase of P450 protein family is nicotinamide adenine dinucleotide phosphate (NADPH)- and O_2-dependent, and displays light reversible co-inhibition (Meehan and Coscia 1973; Collu et al. 2001). G10H also requires a cytochrome P450 reductase (CPR) for its functioning (Meijer et al. 1993a, b). Methyl jasmonate strongly induced G10H expression coordinately with other TIA pathway genes in *C. roseus* cell cultures (Collu et al. 2001). The expression of G10H has been found to be upregulated by cytokinins and ethylene (Papon et al. 2005) and feedback inhibited by TIAs like catharanthine, vinblastine and vindoline (Meijer et al. 1993a, b). 10-Hydroxy-geraniol formed by the action of G10H is further oxidised into 10-oxogeraniol in presence of NADP-oxidoreductase, and the resultant 10-oxogeranial is converted into iridodial by a cyclization reaction by 10-oxogeraniol/iridodial cyclase (Sanchez-Iturbe et al. 2005). The last step of secologanin biosynthesis from loganin involves the oxidative rupture of methylcyclopentane ring catalysed by SLS enzyme. The cloning and functional characterization of SLS have been reported (Irmler et al. 2000). These workers have also observed that this CYP72A1 from *C. roseus* can be heterologously expressed in *Escherichia coli* by converting loganin into secologanin.

The tryptamine and secologanin undergo a condensation reaction to form strictosidine in the cell vacuole. The reaction is known to be catalysed by the enzyme strictosidine synthase (STR) that actually marks the diversion of metabolic flux towards TIA synthesis (De Luca and Cutler 1987; Stevens et al. 1993; de Waal et al. 1995). STR is highly substrate-specific and does not accept loganic acid or tryptophan as substrates. In *C. roseus*, at least seven STR isoforms have been detected (de Waal et al. 1995). Since STR is encoded by a single gene, its isoforms are likely to be the products of posttranslational modifications (McKnight et al. 1990; Pasquali et al. 1999). The mRNA of STR has a region that encodes for a single peptide of 31 amino acids for directing it to vacuole (McKnight et al. 1990, 1991). Strictosidine undergoes a deglucosylation reaction by strictosidine-β-D-glucosidase (SGD) enzyme to yield an unstable aglycon which is a highly reactive ring-opened dialdehyde intermediate in TIA biosynthesis (Geerlings et al. 2000; Barleben et al. 2007). The resultant strictosidine aglycon is spontaneously converted into cathenamine that can either be reduced to form ajmalicine or can also equilibrate chemically with 4, 21-dehydrogeissoschizine that can also be routed towards catharanthine formation via stemmadenine. Ajmalicine is subsequently oxidised to serpentine in vacuole by peroxidases (Blom et al. 1991), and this conversion has been shown to be light-dependent (Loyola-Vargas et al. 1992). Serpentine cannot travel across tonoplast and hence is stored

in vacuoles itself. The SGD enzyme, like many other β-glucosidases, is encoded by a single gene and exists in a supramolecular form to acquire stability (Guirimand et al. 2010b). SGD was earlier postulated to be localised on the outer surface of endoplasmic reticulum (Stevens et al. 1993; Geerlings et al. 2000), but recent observations based on RNA hybridization and green fluorescent protein (GFP) imaging techniques by Guirimand et al. (2010b) have assigned a nuclear localization of SGD. The enzyme was found to be highly specific to strictosidine or its 10-methoxyderivatives and in its native form exists as an aggregate of 63-kDa units linked by hydrophobic interactions (Luijendijk et al. 1996). Post-cathenamine or 4, 21-dehydrogeissoschizine steps towards the synthesis of catharanthine, ajmalicine and tabersonine are largely obscure in *C. roseus*. Only two enzymes, i.e. cathenamine synthase and cathenamine reductase, involved in the initial conversion steps during ajmalicine synthesis have been partially identified (Meijer et al. 1993a, b), but nothing is known regarding their regulation. Both catharanthine and tabersonine are also synthesised in the protoderm and cortical cells of *Catharanthus* root tips (Laflamme et al. 2001), but their synthesis in aboveground parts of the plant is likely to be more compartmentalized as discussed in detail in the subsequent section in this review. Catharanthine has been shown to be exclusively excluded from epidermis and stored in surface wax layer of the leaves (Roepke et al. 2010) as a defence strategy against insect and microbial infestation. In comparison to catharanthine, the biosynthesis of vindoline is better characterized in *C. roseus*. The tabersonine to vindoline conversion is mostly associated with the presence of light and cellular differentiation at different developmental stages of the plants (De Luca et al. 1986; Vazquez-Flota et al. 2002; Campous-Tamayo et al. 2008; Shukla et al. 2010). Much of the interest to dissect vindoline pathway was generated from the fact that undifferentiated cell suspensions and transformed hairy root cultures of *C. roseus* inspite of accumulating high levels of tabersonine generally failed to synthesise vindoline in them (De Carolis et al. 1990; Moreno et al. 1995). The vindoline pathway begins by hydroxylation of tabersonine at C-16 position by the enzyme T16H in leaf epidermis. T16H has been characterized as a P450-dependent monooxygenase (Scroder et al. 1999). The resultant 16-hydroxytabersonine is subsequently converted into 16-methoxytabersonine by 16OMT followed by a hitherto unknown hydroxylase that oxidise it to 16-methoxy-2, 3-dihydro-3-hydroxytabersonine (St. Pierre and De Luca 1995; Levac et al. 2008). Subsequent conversion occurs by a thylakoid membrane-associated 16-methoxy-2, 3-dihydro-3-hydroxytabersonine NMT to yield deacetoxy-vindoline (Dethier and De Luca 1993). Recently, Liscombe et al. (2010) have characterized a *C. roseus* cDNA (Cr2270) encoding an *S*-adenosyl-L-methionine-dependent *N*-methyltransferase that catalyses this nitrogen methylation step. The corresponding gene transcript was found to be induced in methyl jasmonate-elicited seedlings along with other vindoline pathway transcripts. Interestingly, this unique *N*-methyltransferase showed close homology at amino acid level with the plastidial γ-tocopherol C-methyltransferases associated with vitamine E biosynthesis. The last two reactions in vindoline formation are facilitated by D4H (Vazquez-Flota et al. 1997) and DAT (St. Pierre et al. 1998). While T16H and 16OMT are present in sufficient amounts *in planta* as well as corresponding cell cultures, distribution of NMT, D4H and DAT is restricted to laticifer and idioblast cells in the leaves (De Luca and Laflamme 2001; Murata and De Luca 2005; Pasquali et al. 2006; Campous-Tamayo et al. 2008; Shukla et al. 2006, 2010). The NMT is the only enzyme of this pathway whose activity is not affected by light, whereas D4H transcript abundance increased significantly upon exposing the plants to light (Vazquez-Flota et al. 1997; St. Pierre et al. 1998; Vazquez-Flota and De Luca 1998). The earlier observations on cellular compartmentation of enzymes involved in vindoline synthesis in a tissue-specific and age-dependent manner have been recently corroborated with a transcriptome analysis by our group at CIMAP (Shukla et al. 2006, 2010). The differential expression of TIA gene transcripts in the leaf and root tissues of 6-day-, 6-week- and 6-month-old *C. roseus* plants was studied by using a subtractive hybridization approach. The DAT and SGD transcripts were not detected in roots at any developmental stage of the plant, and their expression levels in leaf exhibited a growth-related decrease. This decrease in SGD and DAT transcript levels in leaf was parallel with a concomitant reduction in vindoline content in mature leaf tissues. When SGD and DAT transcript profiling was carried out in methyl jasmonate and *Pythium aphanidermatum* homogenate-elicited and non-elicited undifferentiated cell suspensions, callus and differentiated multiple shoot cultures of *C. roseus*, DAT transcription and presence of vindoline were not evident in cell suspension. Vindoline was also absent in non-elicited as well as methyl jasmonate-elicited callus cultures but appeared upon elicitation by fungal homogenate for 24 h. This also coincided with appearance of DAT and SGD transcripts in such elicited cultures, but the expression levels remained below their levels in the mature plant leaves. These studies substantiated the earlier observation of Rijhwani and Shanks (1998) that level of cellular differentiation and organogenesis and dosage and exposure time of elicitors play a crucial role in patterns of TIA synthesis in *C. roseus*. The fact that SGD transcript level present in undifferentiated cell suspensions in our study (Shukla et al. 2010) was increased to a level higher than those in mature leaf tissue by methyl jasmonate and fungal homogenate elicitation further implied that cellular specialisation is probably not

rigidly mandatory for SGD transcription but necessary for DAT expression.

In contrast to the aerial plant parts of *C. roseus*, the underground roots operate an alternate mechanism for tabersonine metabolism and results in the accumulation of lochnericine and horhammericine (Laflamme et al. 2001). An early step in this route is likely to be conversion of tabersonine into lochnericine by a NADPH- and O_2-dependent tabersonine 6, 7-epoxidase (Facchini and De Luca 2008). This P450 monooxygenase has been found to be associated with microsomes in transformed hairy root cultures of *C. roseus* (Rodriguez et al. 2003). Laflamme et al. (2001) functionally characterized a cDNA for root tip-specific MAT enzyme which is active in root cortical cells and is responsible for the synthesis of 6, 7-dehydroechitovenine and/or 19-*O*-acetylhorhammericine. MAT was shown to have 63% nucleic acid identity and 78% identical amino acid sequences with that of DAT. Co-expression of TDC and STR along with MAT within cortical tissue of the transformed hairy root lines of *C. roseus* also pointed out towards a likelihood of entire TIA pathway for tabersonine synthesis be operational in roots. Ability of MAT to catalyse the 4-*O*-acetylation of deacetylvindoline at very lower efficiency might also explain the presence of trace amounts of vindoline in root cultures of *C. roseus* hairy root cultures in some earlier reports (Shanks et al. 1998; O'Keef et al. 1997).

The dimerization step of vindoline and catharanthine, resulting in the formation of vinblastine and vincristine via α-3', 4'-anhydrovinblastine, marks the completion of TIA pathway in *C. roseus* (Sottomayor and Ros Barcelo 2005; Kumar et al. 2007; Roytrakul and Verpoorte 2007; Costa et al. 2008). At least five isoenzymes of peroxidases have been implicated in this coupling reaction (Endo et al. 1988; Sottomayor and Ros Barcelo 2003; Sottomayor et al. 1998, 2004). One of these class III basic peroxidases namely α-3', 4'-anhydrovinblastine synthase (AVLBS) has been purified to homogeneity from *C. roseus* leaves with a molecular mass of 45.4 kDa and pH optima of 6.5 (Sottomayor et al. 1998). The AVLBS, commonly known as Prx1, is consists of 363 amino acids including an N-terminal signal polypeptide for targeting it to vacuole (Costa et al. 2008). The Prx1 gene is encoded by a single copy gene with two introns. The size of the full-length Prx1 fluctuates between 1,216 and 1,370 bp due to the presence of various polyadenylation sites (Sottomayor et al. 2004). The expression of Prx1 mRNA is discernible right after 6 days of seed germination in *C. roseus* and can be detected in all aerial tissues, but not in roots. The basipetal expression of Prx1 in leaves follows a pattern similar to other TIA pathway genes, i.e. high expression in very young leaves, decreasing expression in fully expanded leaves and eventually increasing again during senescence (Costa et al. 2008). Some workers have also proposed that an iminium ion may be formed as an unstable intermediate during the dimerization step, and both anhydrovinblastine and iminium ion can be incorporated into vinblastine and vincristine (Gueritte et al. 1980; Sottomayor et al. 2004). Recently, three more class III basic peroxidases have been characterized in *C. roseus* (Kumar et al. 2007; Jaggi et al. 2011). One of these novel peroxidases, namely CrPrx (Acc. No. AY924306), unlike vacuolar Crx1, was found to be apoplastic in nature and ubiquitous in all plant parts except in very young leaves. The CrPrx nucleotide sequence encodes a deduced translation product of 330 amino acids with a 21-amino-acid signal peptide which implied its secretory nature (Kumar et al. 2007). Since CrPrx showed normal transcript level in roots, it was further prospected to ascertain its role in production of root-specific TIAs in transformed hairy root cultures of *C. roseus* (Jaggi et al. 2011). Root clones overexpressing CrPrx gene under the control of a CaMV35S promoter indicated 1.8–8.7-fold increase in its transcript levels along with higher H_2O_2 production. qRT-PCR analysis of these clones also showed differential transcript profiles of TDC, G10H and SGD. A 2–4-, 2.5–15- and 1.3–6.5-fold increase in mRNA transcripts of these three respective TIA pathway genes was evident in the analysed roots. However, transcript profiling of zinc finger protein and G-box binding factor, that are known to act as repressor of TIA synthesis, showed a marked decrease and enhanced transcript accumulation, respectively. When these data were contrasted with metabolite accumulation pattern in hairy root clones, it was observed that ajmalicine levels were more or less stable, but serpentine accumulation was greatly enhanced in all CrPrx overexpressing clones. The high ajmalicine-producing clone also showed concurrent upregulation of STR and SGD genes. Interestingly, expression of Prx1 gene, which is otherwise known to be absent or present at a low level in roots, also increased in CrPrx-RNAi transgenic root line. This research group has recently reported the cloning and characterization of two more novel peroxidase genes, CrPrx3 and CrPrx4, in *C. roseus* (Kumar et al. 2011). The cDNA of these novel enzymes are 1,233 and 1,055 bp long encoding 330 and 318 amino acid residues. Both the peroxidases are apoplastic in nature and have maximum expression in stem tissues followed by flowers.

Temporal and spatial organization of TIA pathway: new insights into the complexities of an old dilemma

Intercellular compartmentation of TIA pathway

Ever since the initial breakthrough in the identification and localization of biosynthetic enzymes by histochemical

staining techniques in 1980s to mid-1990s (see reviews by De Luca and Cutler 1987; Kutchan 1995; van der Heijden et al. 2004), the TIA pathway has remained in focus of extensive scientific scrutiny because of its differential expression in different plant organs in a time- and age-dependent manner. The dynamics of the TIA assembly and accumulation clearly suggested that this pathway is far more than a straightforward linear arrangement of consecutive enzymatic reactions (Pasquali et al. 2006; Facchini and De Luca 2008). The information gathered following the interventions of modern in situ RNA hybridization and immunolocalization tools in pathway elucidation efforts has pointed out that the execution of this pathway is under rigid ontogenic, cellular/subcellular and ecophysiological controls (Kutchan 2005; Guirimand et al. 2010a). The first comprehensive effort made in this direction by St. Pierre et al. (1999) led to the prediction of a three cell-type model for this pathway elaboration that sequentially involves: the internal phloem-associated parenchyma (IPAP), the epidermis and the specialised laticifers and idioblast cells for operating different portions of TIA pathway in the aerial tissues of *C. roseus* (Fig. 2). The IPAP cells present in the periphery of stem pith or intraxylary on the upper part of the vascular bundles in leaves (Mahroug et al. 2007) are primary locations for the expression of early pathway genes like DXR, DXS, MECS and HDS of MEP pathway along with G10H (Burlat et al. 2004). The epidermis, on the other hand, harbours the maximum expression of SLS, TDC and STR gene products (St. Pierre et al. 1999; Irmler et al. 2000), and idioblast and laticifer cells embedded in the palisade tissue of the leaves represent the exclusive location of D4H and DAT gene activities. These TIAs pathway genes, under normal physiological conditions of the plant, followed a gradient trend of expression, i.e. highest expression in younger leaf cells present near the point of attachment with nodes and gradually tapering towards fully matured tissue towards the leaf tips. In the underground root tissue, TDC, STR and MAT transcripts are localised in protoderm and cortical cells around root apical meristem (Laflamme et al. 2001; Moreno-Valenzuela et al. 2003). The existence of such a multicellular model for TIA biosynthesis obviously implies that for maintaining the continuity of the metabolic flux, it is essential that a hitherto unknown intermediate is transferred from IPAP to epidermis, and another unknown intermediate is retranslocated from epidermis to idioblast and laticifer cells of the leaf (St. Pierre et al. 1999). Since a large number of studies on transformed hairy root cultures of *C. roseus* have shown the accumulation of catharanthine and tabersonine along with their substituted derivatives lochnericine and horhammericine in the cortical cells of the roots (Bhadra et al. 1993; Morgan and Shanks 1999; Laflamme et al. 2001; Magnotta et al. 2007), it is still not clear whether biosynthesis of catharanthine occurs independently in the leaf epidermis and roots or this monomeric TIA is synthesised in roots and then sent to aerial tissues for its dimerization with vindoline. Alkaloid quantification in rootless shoot cultures of *C. roseus* (Hirata et al. 1990; Vazquez-Flota and De Luca 1998; Hernandez-Dominguez et al. 2004; Shukla et al. 2010) showing the presence of catharanthine, however, favours the first possibility.

Many puzzling complexities related to TIA biosynthesis are now being resolved with the help of Laser Capture Microdissection (LCM) and Carborundum Abrassion (CA) techniques by the research group of Prof. De Luca (Murata and De Luca 2005; Murata et al. 2008; Levac et al. 2008; Roepke et al. 2010). The LCM technique has allowed the laser-mediated precise harvesting of different cell types from 150–300-μm-thick longitudinal, transfer and paradermal sections of the plant tissues, followed by isolation of enriched mRNA from them (Murata and De Luca 2005). On the other hand, CA was employed as a complementary approach to obtain epidermis-enriched leaf extracts to monitor enzyme and gene expression profiles in the backdrop of alkaloid quantification. Precise targeting of cells by LCM technique was facilitated by their distinct appearance under microscope. For example, the cross-connected laticifers were targeted by their unique canal-like appearance in paradermal section, whereas palisade-assisted idioblasts were distinguishable by their rounded shape and larger size than adjoining palisade mesophyll in such sections. The vascular cells appeared as darker and tighter tissue amongst loose thin-walled mesophyll in the transverse sections. The mRNA harvested from enriched populations of each of the four cell types was subjected to T7RNA amplification before reverse transcription polymerase chain reaction (RT-PCR) analysis with primers specific to nine pathway genes. Results of TDC, STR, D4H and DAT followed an expression pattern consistent with those obtained in in situ hybridization experiments of St. Pierre et al. (1999) and established that while TDC and STR are expressed in epidermal cells, D4H appeared in epidermis, idioblast and laticifer and DAT in laticifer cells. In addition, T16H and SGD were also found to be expressed in the epidermis, whereas ORCA3 and an AP2/ERF type of transcription factors that were earlier shown to regulate the expression of TDC, STR and D4H (van der Fits and Memelink 2001) were expressed in all four cell types tested by Murata and De Luca (2005). The expression of G10H which was earlier suggested to be confined to vascular tissue (Burlat et al. 2004), was found to be present in epidermis and laticifer in LCM-based RT-PCR results. In the CA technique, the upper and lower epidermis from young *C. roseus* leaves was abraded with carborundum F with the help of a cotton swab. The rubbed epidermis was dipped in protein or alkaloid extraction solutions to produce

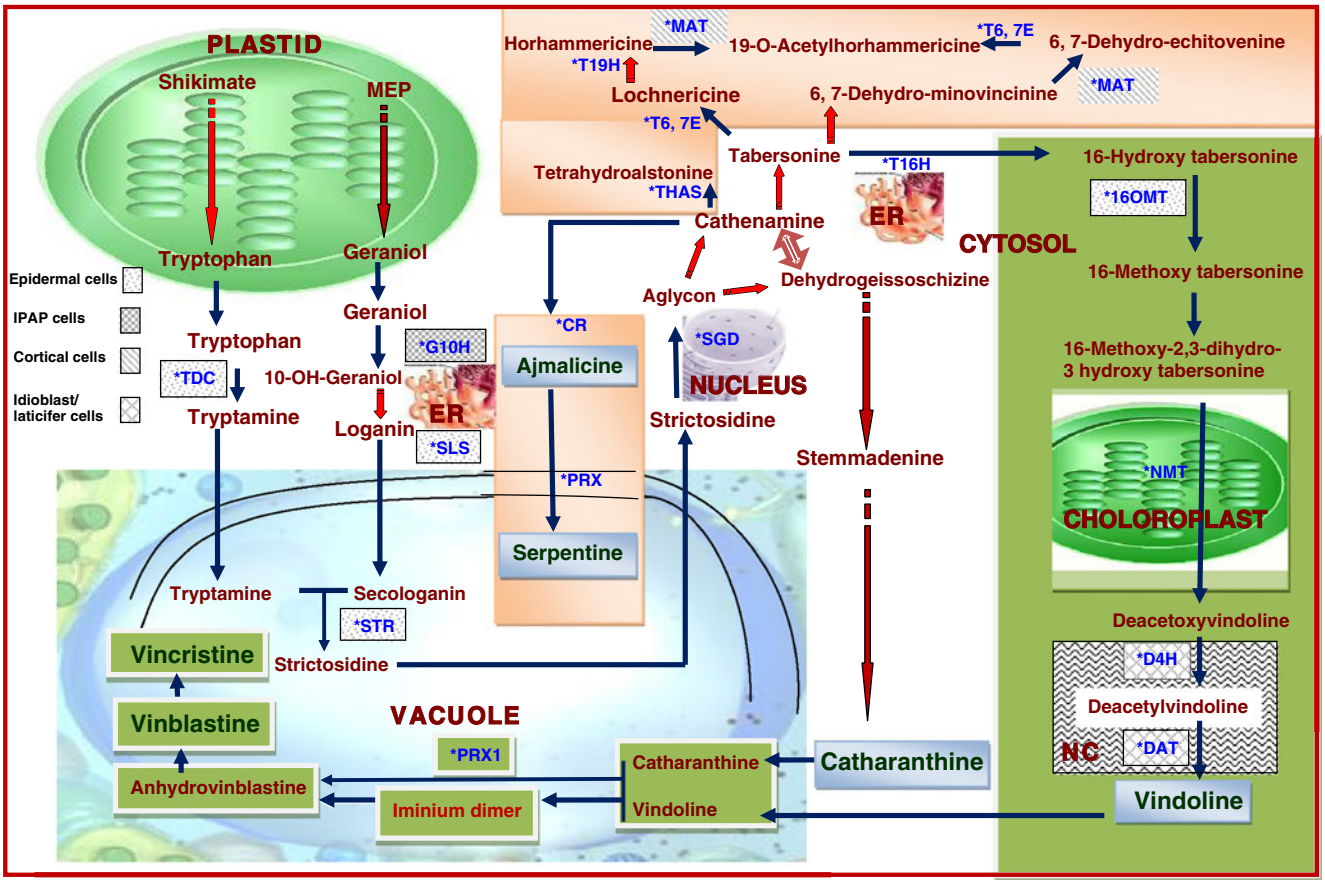

Fig. 2 The spatial inter- and intracellular organization of terpenoid indole alkaloid pathway in different plant parts/cells of *C. roseus*. *Green boxes* indicate the leaf-specific and *orange boxes*, the root specific steps; *Red broken arrows* represent multi-step or uncharacterized reactions; *ER* endoplasmic reticulum, *NC* nucleocytoplasm, *MEP* methylerythritol phosphate, *IPAP* internal phloem-associated parenchyma, *denotes biosynthetic enzymes [*TDC* tryptophan decarboxylase, *G10H* geraniol-10-hydroxylase, *SLS* secologanin synthase, *STR* strictosidine synthase, *SGD* strictosidine β-glucosidase, *T16H* tabersonine 16-hydroxylase, *16 OMT* 16-hydroxytabersonine-16-*O*-methyltransferase, *NMT N*-methyltransferase, *D4H* deacetoxyvindoline-4-hydroxylase, *DAT* deacetylvindoline 4-*O*-acetyltransferase, *T6,7E* tabersonine 6,7-epoxidase, *T19H* tabersonine 19 hydroxylase, *MAT* minovincinine-19-*O*-acetyltransferase, *CR* cathenamine reductase, *PRX1* peroxidase, *THAS* tetrahydroalstonine reductase]

crude epidermal extracts. Alkaloid analysis of CA extract showed very low levels of vindoline and catharanthine (0.16–1.8 of total TIAs) in comparison to whole leaf extracts, suggesting their likely accumulation in the central part of the leaf, i.e. palisade, laticifer, idioblast and vasculature. At the same time, the epidermal extract showed 5 and 11 times higher contents of tabersonine and 16-methoxytabersonine, respectively, when compared with whole leaf (Murata and De Luca 2005; Murata et al. 2008). TDC activity was maximum in the epidermal cells, while DAT activity was detected only in the whole leaf extract. High 16-OH OMT activity was also detected in both abaxial and adaxial epidermal cells than in the whole leaf, whereas the NMT activity in the whole leaf extract again supported its localization in the chloroplast thylakoids. The enzyme activity profile showed a matching trend with RT-PCR data of RNA analysis of TDC, G10H, SLS, STR, SGD, T16H, D4H, DAT and ORCA3 in abaxial and adaxial epidermal cells. The presence of mRNA of STR and SGD in the epidermal cells also pointed out that both strictosidine formation and its deglucosylation occur in the same cell. The presence of mRNA of both G10H and SLS enzymes in leaf epidermis also pointed out that epidermis might also be the site for secologanin biogenesis in addition to vascular cells where G10H was previously shown to be localised (Burlat et al. 2004). As mentioned earlier, the leaf epidermis of *C. roseus* was also a site of expression of PAL, C4H and CHS genes of phenylpropanoid pathway (Mahroug et al. 2006; Murata et al. 2008). Expression of PAL, C4H and CHS genes along with SLS, TDC and STR genes of TIA pathway in the same epidermal cell of *C. roseus* leaves led these workers to propose a common transcriptional control of these genes. The CA approach also led to the generation of a leaf epidermome-enriched cDNA library to identify and functionally characterize several of the genes associated with this pathway (Murata et al. 2008). A

population of 3655 expressed sequence tags (ESTs; CROLF1NG data set) was found to contain essentially all the known TIA pathway genes that were shown to be expressed in *C. roseus* leaf epidermis. The 3655 unique sequences of this ESTs data set were composed of 1142 clusters and 2513 singletons. Detailed analysis of this data set clearly revealed its richness for genes like 10, hydroxygeraniol oxidoreductase, SLS, CPR, TDC, STR, SGD, T16H, ORCA3, box P binding factor 1 and zinc finger *Catharanthus* transcription factor 2. Abundance of LAMT transcripts along with one EST of G10H in this data set also indicated that complete secologanin biosynthesis is expressed in epidermis. The situation will, however, remain ambiguous till the issue regarding absolute necessity of transportation of a hitherto unknown isoprenoid intermediate from IPAP cell to epidermis for secologanin formation is fully settled through further experimentation. Two additional candidate ESTs in the CROLF1NG data set for 7-deoxyloganetic acid1-*O*-glucosyltransferase and 7-deoxyloganin-7-hydroxylase enzymes also await detail gene cloning and characterization experiments.

Recent studies on catharanthine accumulation in *C. roseus* have also shown that most of these monomeric TIAs are present as exudates in wax layer of the leaf surface (Roepke et al. 2010). This probably serves two purposes: physical separation of catharanthine from vindoline (present in laticifers and idioblasts) to restrict their coupling to form cytotoxic/antimitotic dimeric alkaloids vinblastine and vincristine, unless required by the plant; and catharanthine on surface wax layer could act either as a strong deterrent to insect herbivory or fungal infection. These workers have experimentally shown that chloroform-soluble fraction of leaf surface contained nearly 100% of catharanthine content along with 3–5% of vindoline in comparison to the whole leaf extract. The accumulation and secretion of catharanthine in leaf wax layer again pointed out the likelihood of a complete catharanthine biosynthetic pathway to be operative in leaf epidermis. Roepke et al. (2010) further studied the antifungal activity of catharanthine against *Phytopthora nicotianiae*. Catharanthine was found to inhibit the fungal growth at a concentration of 10 μg/ml in the culture medium, which was far below its average concentration of 23 μg and 14 μg/cm^2 leaf surface in young and older leaves. The anti-herbivory effect of catharanthine was also prospected by feeding the *Catharanthus* leaves to *Spodoptera littoralis*, *Spodoptera eridania*, *Helicoverpa armigera*, *Phaedon cochleariae* and *Bombyx mori*. The results showed that third star larvae of *S. eridania* did not eat *Catharanthus* leaves, whereas the two *Spodoptera* species as well as *B. mori* consumed varied amount of leaves without any incident of death. In contrast, *P. cochleariae* refused to accept *Catharanthus* leaves and died of starvation within 5 days. The sixth star larvae of *B. mori* also showed progressive death in a dose-dependent manner when fed with standard mulberry diet mixed with varying amounts of pulverised whole leaf or catharanthine-enriched leaf surface extracts. The incidence of death was delayed when whole pulverised leaf extract was prepared after catharanthine and other surface compounds were removed by chloroform treatment. On the other hand, when larvae were fed with mulberry diet containing different amounts of pure catharanthine, they again died progressively in a dose-dependent manner.

Subcellular compartmentation of TIA pathway genes and enzymes

Like their spatial and temporal organization, the subcellular expression and trafficking of TIA pathway enzymes and intermediates also revealed a complex inter-organelle exchange mechanism in *C. roseus* (van der Heijden et al. 2004; Facchini and St. Pierre 2005; Mahroug et al. 2007; Ziegler and Facchini 2008). Nevertheless, only a few enzymes and intermediates have so far been characterized in this direction. Besides cytosol, five subcellular compartments have been implicated in this mechanism namely: plastids, vacuole, endoplasmic reticulum, mitochondria and nucleus (Fig. 2). While earlier studies in this area mainly relied on density gradient analysis, past few years have witnessed the increasing use of immunogold staining, GFP-fusion imaging, in situ hybridization, *in silico* modelling and transient transformation approaches to precisely assign the molecular structure and subcellular localization of various TIA enzymes (Costa et al. 2008; Guirimand et al. 2009; 2010a, b; 2011a, b). TDC, OMT, D4H and DAT enzymes essentially operate in the cytosol (De Luca and Cutler 1987; Vazquez-Flota et al. 1997). This implies that tryptophan synthesised in the plastids via shikimate pathway has to move out in the cytosol for its decarboxylation by TDC to yield tryptamine. Tryptamine, because of its cytotoxic effect on cellular metabolism, is immediately transported to cell vacuole for its subsequent condensation with secologanin. For secologanin biosynthesis, the geraniol derived from plastidial MEP pathway is guided towards vacuole following a hydroxylation reaction by G10H enzyme. G10H which was earlier believed to be a pro-vacuolar membrane enzyme (Madyastha et al. 1977) has now been conclusively assigned an endoplasmic reticulum (ER) site of localization (Guirimand et al. 2009). Using a refined particle bombardment-assisted transient transformation approach in combination with GFP imaging by epi-fluorescence microscopy, these workers have clearly demonstrated the ER localization of G10H-GFP plasmid construct in C20A cell line of *C. roseus*. This ER localization of the fusion protein was supported by the presence of a predicted 20-residue transmembrane helix at N-terminal end of G10H. Fusion of this N-terminal domain

to GFP and deletion from G10H reinforced the inference that this 20-residue helix was necessary for the ER anchoring and led to the exposure of its carboxyl terminal tail towards the cytosol. This proposed model of G10H localization in ER is also consistent with subcellular localization of cytochrome P450 reductase in ER for electron transfer to cytochrome P450 enzymes. The research group of Professor Guirimand has also recently settled the uncertainties regarding the localization of another secologanin pathway enzyme namely secologanin synthase (Guirimand et al. 2011b). SLS, which is also a cytochrome P450 family enzyme, was earlier shown to lack a putative C-terminal ER membrane anchoring signal by Irmler et al. (2000) and was thought to be localised in tonoplast on the basis of the presence of its substrate (loganin) and product (secologanin) in the vacuole (Contin et al. 1999). Using RNA in situ hybridization approach in combination with binocular fluorescence complementation assays and yeast two-hybrid analysis, Guirimand et al. (2011b) have reported that both TDC and LAMT form homodimers in the cytosol, whereas SLS is anchored to endoplasmic reticulum via an N-terminal helix. Consequently, secologanin and tryptamine are transported to vacuole to facilitate the synthesis of strictosidine. In addition to G10H localization in ER, Guirimand et al. (2009) were also able to indicate the subcellular occurrence of hydroxymethyl-butenyl-4-diphosphate synthase (HDS) enzyme of MEP pathway in the long stroma-filled thylakoid-free extensions (stromules) budding from plastids. The co-transformation of G10H-GFP and HDS-YFP constructs, which showed a close association between cortical ER and stromules, suggested a possible way to export MEP pathway products to ER-anchored G10H for hydroxylation step to occur. Two more TIA pathway enzymes that have received a lot of attention in inter- and intracellular localization and expression studies are STR and SGD that are associated with the biosynthesis of strictosidine and its subsequent deglucosylation to form the unstable aglycon, respectively. STR was earlier reported to be localised either to the cytoplasm or to the vacuole (De Luca and Cutler 1987; McKnight et al. 1991; Stevens et al. 1993), but finally, a vacuolar localization is now proved (Guirimand et al. 2010a). The appearance of STR-GFP signal in the vacuole in transient transformation experiment was in agreement with the predicted N-terminal signal peptide followed by a vacuolar sorting-like sequence. The N-terminal truncated version of STR deprived of this signal peptide failed to enter the secretory pathway to reach the vacuole. As a result, the fusion product gets accumulated in the cytoplasm with a passive diffusion to nucleus. Treatment with Brefeldin A, a drug that disrupts the ER-to-cis Golgi endomembrane transport system, led to the retention of STR-GFP within the disorganised ER, proving thereby that the vacuolar targeting of STR is achieved via an ER-to-Golgi-to-vacuole route.

SGD was first indirectly hypothesised to be localised in ER on the basis of in vivo apparition of strictosidine-induced yellow fluorescence in ER and presence of a putative ER-anchoring KKXKX C-terminal sequence (Geerlings et al. 2000). However, Guirimand et al. (2010a, b), in their GFP imaging experiments, contradicted these observations by reporting that SGD-GFP is co-localised with the nucleus marker and was excluded from the ER. A C-terminal bipartite nuclear localization signal (NLS) was also identified that included the putative KKXKX C-terminal sequence observed in the previous study by Geerlings et al. (2000). Interestingly, in the reverse fusion experiment, the construct without NLS accessible GFP-SGD was also targeted to the nucleus, but with a punctuated fusiform fluorescence pattern with increased expression time. The SDS–PAGE and anti-GFP western blotting data of cell lines of *C. roseus* transformed with SGD-GPF and GPF-SGD constructs, respectively, again displayed the similar diffuse versus aggregated nuclear fluorescence patterns as were obtained in transient transformation experiments. Recovery of a 91-kDa band with both SGD-GFP and GFP-SGD constructs was consistent with the fusions based on 63 kDa of SGD and 28 kDa of GFP. This comprehensive SGD localization work clearly suggested that SGD is targeted to nucleus using a bipartite NLS and tends to multimerize in this cellular compartment. This unusual localization of SGD in the nucleus may be essential for its physical separation from accumulated strictosidine pool in the vacuole under normal physiological conditions. A massive activation of strictosidine pool may occur upon organelle disruption during herbivore feeding or necrotrophic pathogen attack in a “nuclear time bomb” fashion (Guirimand et al. 2010b). The induced protein cross-linking and precipitation by resultant aglycon in this process might also be a powerful defence strategy of *C. roseus* plants to deter herbivores from feeding them. Expression of both STR and SGD activities in epidermal layer of *C. roseus* leaves further substantiate this first-line defence mechanism in *C. roseus*.

As discussed in the previous section, the subcellular localization of pathway enzymes involved with vindoline synthesis and their dimerization reaction with catharanthine to form vinblastine and vincristine are fairly well understood now. In brief, T16H is anchored to ER as a monomer (St. Pierre et al. 1999), whereas 16OMT is found to homodimerize in the cytoplasm to facilitate the uptake of T16H conversion product. NMT is present in the thylakoid membrane of the chloroplasts (De Luca and Cutler 1987; Dethier and De Luca 1993), and D4H and DAT that were largely believed to operate in the cytosol of idioblast and laticifer cells have now been shown to operate as monomers and reside in nucleocytoplasmic compartment because of their passive diffusion to nucleus due to smaller protein size

(Guirimand et al. 2011a). Finally, the vacuolar localization of peroxidases (AVLBS/Prx1) associated with the coupling of catharanthine and vindoline to form bisindole alkaloids is also well established now.

Conclusion and future prospects

Intervention of powerful tools of modern plant biology and chemistry in the last two decades has advanced our current understanding of TIA pathway metabolism in *C. roseus* to a level where several well-planned metabolic engineering approaches to boost their *in planta* or in vitro production can be foreseen. Gene manipulations around geraniol, chorismate, tabersonine/vindoline and catharanthine subways will constitute important targets for these attempts. It is also envisaged that rapid strides will be made in the identification and functional characterization of unknown pathway enzymes, intermediates and genes to understand the linearity/diversions in this metabolic route under different sets of developmental, environmental and stress signals. Since rigid inter- and intracellular organization of major TIA pathway enzymes is now well documented, mechanisms controlling the translocation of metabolites between different plant organs, tissues, cells and cell organelles will also constitute an area of intense scientific efforts in *C. roseus* biology. Members of ATP-binding transporter family will occupy a centre stage in this prospection. Parallel progress will also be launched to understand the global transcription regulation of TIA pathway genes beyond ORCA3. It is hoped that recent breakthrough from our laboratory (Verma and Mathur 2011a, b) towards the development of efficient methods of regeneration and transgenic plant production in *C. roseus* will speedup these developments.

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Conflict of interest The authors declare that they have no conflict of interest.

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