Metabolomic characteristics of Catharanthus roseus plants in time and space

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Chapter 2

Terpenoid indole alkaloids biosynthesis and its regulation in *Catharanthus roseus*: a literature review from genes to metabolome

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Abstract

As the only source for the antitumor agents vinblastine and vincristine, *Catharanthus roseus* is highly valued as supply of pharmaceuticals. As the production of these alkaloids is very low, the biosynthesis of these terpenoid indole alkaloids (TIAs) has been studied extensively as a model for medicinal plant's improvement. The TIA pathway is a complex multistep enzymatic network that is tightly regulated by developmental and environmental factors. Here we review the knowledge achieved in the past 30 years of the TIA pathway in *C. roseus*, from genetic to metabolic aspects. Two early precursor pathways (chorismate-indole pathway and MEP-secoiridoid pathway) and late mono-/bis-indole alkaloid pathways have been largely elucidated and established, as well as their intercellular and subcellular compartmentation. Many genes encoding constitutive structural biosynthetic enzymes, transcription factors, and transporters involved in these pathways have been cloned and characterized. These genes were applied in metabolic engineering strategies to improve the production of TIAs. However, modification at genetic/molecular level of the pathway in *C. roseus* resulted in complicated changes of metabolism, not only the TIA pathway was affected, but also other pathways, both in secondary and primary metabolism. Research at metabolic level is required to increase the knowledge on the genetic regulation of the whole metabolic network connected to the TIA biosynthesis. Metabolomics, as a powerful tool for qualitative and quantitative analysis at metabolic level, provides a comprehensive insight into the metabolic network of the plant system. Metabolic profiling and fingerprinting combined with multivariate data analysis, metabolic flux analyses based on ^{13}C labeling experiments in combination with other "omics" have been implemented in studies of *C. roseus* for pathway elucidation, including among others, understanding stress response, cross talk between pathways, and diversion of carbon fluxes, with the aim to fully unravel the TIA biosynthesis, its regulation and the function of the alkaloids in the plant from a system biology point of view.

Introduction

Terpenoid indole alkaloids (TIAs) form a major group of alkaloids in the plant kingdom, comprising of over 3000 identified alkaloids to date. The TIAs have structures derived from strictosidine formed from tryptamine and a C_{10} part from the iridoid secologanin. Many of these natural products are physiologically active in mammals. But they are found to be confined to eight different plant families (e.g., the most important ones: Apocynaceae, Loganiaceae, Rubiaceae.). Several pharmaceuticals belonging to the TIA group are commercially isolated from plant materials (Table 1), like the antimalarial alkaloid quinine from *Cinchona officinalis*, the antineoplastic camptothecine from *Camptotheca acuminate*, the rat poison and homeopathic strychnine from *Strychnos nux-vomica*, reserpine from *Rauvolfia* species and the antitumor agents vinblastine and vincristine from *Catharanthus roseus* (periwinkle) (Kutchan, 1995).

Catharanthus roseus, belonging to the Apocynaceae family, is a medical plant of great pharmaceutical interest for its capacity to biosynthesize a great variety of TIAs (>130), which have a high economic value due to their wide spectrum of pharmaceutical applications. Besides the most well-known bisindole alkaloids (vinblastine and vincristine), *C. roseus* also produces ajmalicine used as antihypertensive and serpentine used as sedative. The very small amounts of the dimeric alkaloids in *C. roseus* and the difficulty of their extraction and purification explain the high costs of these TIAs. Although total chemical synthesis of these complex alkaloids is of academic interest, this is not likely to be applied commercially due to the low yields. However, the dimerization reaction coupling vindoline and catharanthine, which in the plant is catalyzed by a peroxidase, has been mimicked chemically and is now used to couple the much more abundant monomers. The *in vitro* production systems using plant cell cultures or hairy roots of *C. roseus* have been developed but failed to synthesize vindoline, one of the precursors needed for the bisindole alkaloids. To develop novel sources of these compounds requires thorough knowledge of genes, enzymes, intermediates in the TIA biosynthetic pathway and the regulation mechanism behind it.

Table 1 Active TIAs, their functions and plants that produce them.

Alkaloids	**Function**	**Plants**
Ajmalicine	antihypertensive	*Catharanthus roseus*
Camptothecine	antineoplastic	*Camptotheca acuminate*
Ellipticine	antitumour	*Ochrosia elliptica*
Emetine	anti-protozoal	*Carapichea ipecacuanha*
Quinidine	class I antiarrhythmic agent (Ia) in the heart	*Cinchona* spp.
Quinine	antimalarial alkaloid	*Cinchona* spp.
Rescinamine	antihypertensive	*Rauwolfia* spp.
Reserpine	antipsychotic, antihypertensive	*Rauwolfia serpentina*
Serpentine	sedative	*Catharanthus roseus*
Strychnine	rat poison and homeopathic	*Strychnos nux-vomica*
Toxiferine	curare toxin, muscle relaxant	*Strychnos toxifera*
Vinblastine, Vincristine	antitumor	*Catharanthus roseus*
Vincamine	peripheral vasodilator that increases blood flow to the brain	*Vinca minor*
Yohimbine	mild monoamine oxidase inhibitors with stimulant and aphrodisiac effects	*Corynante Yohimbe*

In the past decades relentless efforts and meticulous in-depth studies were carried out on TIAs in *C. roseus* by numerous groups of researchers all across the globe. Nowadays the "Omics" tools, such as genomics, transcriptomics, proteomics and metabolomics, provide us with enormous amounts of information about the genes, enzymes, transcription factors, intermediates, pathways, and compartmentation of TIA biosynthesis in *C. roseus* cell cultures, hairy roots and plants. These tools will be very helpful to clarify some unresolved parts of the iridoid pathway, the catharanthine biosynthesis, transport, and the signal-transduction and regulation of the pathway via transcription factors like the octadecanoid-responsive *Catharanthus* AP2-domain (Orca's).

The latest developments in the studies of the biosynthesis of TIA and its regulation in *C. roseus* by genomic and metabolomic studies are reviewed in the present update.

Alkaloid biosynthesis and its compartmentation in *Catharanthus roseus*

TIA biosynthesis in *C. roseus* is a complex pathway including at least 30 coordinately regulated enzymatic steps producing at least 35 known intermediates. The pathway is spread over at least 4 different cell types and in these cells at least 5 different subcellular compartments are involved, which means that transport is a major rate determining factor, which besides physical chemical principles (e.g. diffusion, mass transfer between two phases) also involves different types of active selective transporter proteins (Roytrakul *et al.*, 2007). Up to now, 30 biosynthetic and 4 types of regulatory genes have been cloned and identified. However, there are still unknown parts of TIA biosynthesis to be solved. Alkaloids in *C. roseus* derive from the convergence of two primary metabolic routes, i.e. the shikimate and the secoiridoid pathways that respectively provide the indole and the terpene moiety to the basic backbone as found in strictosidine (van der Heijden *et al.* 2004; Verma *et al.*, 2012).

The shikimate-chorismate-indole pathway and its localization

The shikimate pathway, a major biosynthetic route for both primary and secondary metabolism, starts with phosphoenolpyruvate and erythrose-4-phosphate and ends with chorismate (Herrmann and Weaver, 1999) (Fig. 1). Chorismate is an important starting point and the substrate of 5 enzymes that are the gatekeepers of the five pathways that branch from chorismate: anthranilate synthase (AS) on the branch leading to tryptophan; chorismate mutase (CM) on the branch leading to phenylalanine and tyrosine; isochorismate synthase (ICS) on the branch leading to isochorismate, salicylate and 2,3-dihydrobenzoic acid (2,3-DHBA); chorismate pyruvate-lyase synthase (CPL) on the branch to p-hydroxybenzoate (a precursor of shikonin); and

p-aminobenzoate synthase on the branch to folates (Mustafa and Verpoorte, 2005).

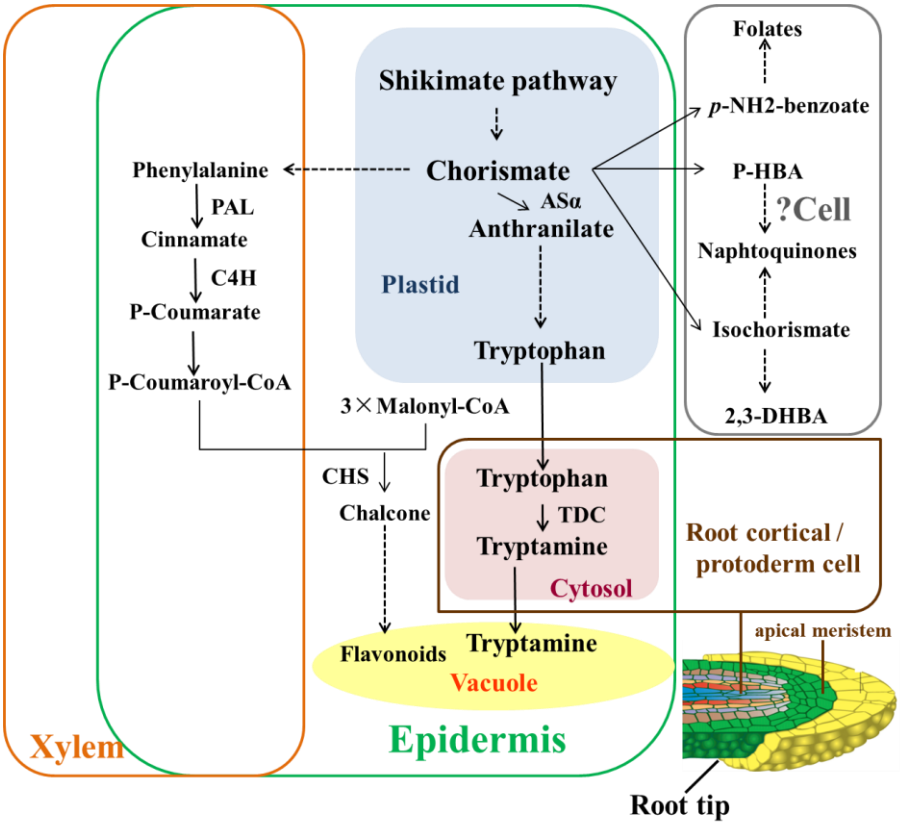

Fig. 1 The shikimate-chorismate-indole pathway and its compartmentation at intercellular and subcellular level. Solid arrows represent one-step reactions; broken arrows represent multiple or uncharacterized reactions.

The anthranilate pathway leads to the formation of the aromatic amino acid tryptophan, which provides the indole moiety to TIAs (Fig. 1). Anthranilate synthase (AS) is the first key enzyme in the synthesis of tryptophan and indole-3-acetic acid, which consists of two large (α-subunits) and two small (β-subunits) subunits (Poulsen *et al.*, 1993). The α-subunit of AS is responsible for catalyzing the conversion of chorismate into anthranilate and is sensitive to tryptophan-mediated feedback inhibition (Verpoorte *et al.*, 1997; Radwanski

and Last, 1995). But after AS, none of the enzymes leading to tryptophan have been studied in *C. roseus* so far (El-Sayed and Verpoorte, 2007). Tryptophan is conversed into tryptamine by tryptophan decarboxylase (TDC) (Noe *et al.*, 1984). The genes of both AS (Hong *et al.*, 2006) and TDC (Canel *et al.*, 1998; Goddijn *et al.*, 1995; Whitmer *et al.*, 2002b) have been cloned and overexpressed in cell cultures or hairy roots of *C. roseus.*

In the aerial tissues, the epidermis of leaf harbors the maximum expression of *TDC* products. In the underground tissues, *TDC* transcripts are localized in protoderm and cortical cells around the root apical meristem (St. Pierre *et al.*, 1999; Irmler *et al.*, 2000). The early steps of the tryptophan pathway are thought to occur in plastids (Zhang *et al.*, 2001). The TDC enzyme essentially operates in the cytosol (De Luca and Cutler, 1987). This implies that tryptophan has to move out from plastids to the cytosol for its decarboxylation by TDC to yield tryptamine, which is immediately transported to the cell vacuole for its subsequent condensation with secologanin (Fig. 1). The leaf epidermis of *C. roseus* is also a site of expression of PAL, C4H and CHS genes of the phenylpropanoid pathway (Mahroug *et al.*, 2006; Murata *et al.*, 2008).

The MEP-secoiridoid pathway and its localization

The terpenoid moiety of TIAs comes from secologanin biosynthesized via the secoiridoid pathway, which derives from a universal precursor for all monoterpenes geranyl pyrophosphate (GPP). There are two separate pathways in plants to produce isopentenyl diphosphate (IPP), the central precursor of all isoprenoids. One is the mevalonate pathway leading to the formation of triterpenes (sterols) and certain sesquiterpenes (Newman and Chappell, 1999; Lange and Croteau, 1999). The other is the mevalonate-independent (MEP) pathway leading to the formation of monoterpenes, diterpenes, tetraterpenes (carotenoids) and the prenyl-sidechains of chlorophyll (Eisenreich *et al.*, 1996; Arigoni *et al.*, 1997; Rohmer, 1999). ^{13}C labeling experiments followed by NMR proved that the MEP pathway is the major route for the biosynthesis of secologanin in *C. roseus* (Contin *et al.*, 1998).

The MEP pathway leading to the formation of isopentenyl diphosphate (IPP) has been unraveled in plants. A complete set of MEP pathway genes in *Arabidopsis* homologous to the corresponding genes in *E. coli* have been

identified (Ganjewala *et al.*, 2009). The MEP pathway involves 7 enzymatic steps, starting from the condensation of pyruvate with D-glyceraldehyde 3-phosphate ending with the production of IPP (El-Sayed and Verpoorte, 2007). The pathway starts with the enzyme 1-deoxy-D-xylulose-5-phosphate synthase (DXS) that catalyzes the condensation of pyruvate with D-glyceraldehyde 3-phosphate to 1-deoxy-D-xylulose-5-phosphate, the first step of the MEP pathway. This product is then converted into 2-C-methyl-D-erythriol-4-phosphate by the enzyme 1-deoxy-D-xylulose-5-phosphate reducto isomerase (DXR) (Fig, 2).

The final product IPP is converted to dimethylallyl diphosphate (DMAPP) by isopentenyl diphosphate isomerase (IDI). The encoding gene was cloned from many sources including from *C. roseus* (*CrIDI1*) (Guirimand *et al.*, 2012). This enzyme, previously abbreviated as IPPI (Ramos-Valdivia *et al.*, 1997), is expressed in all organs (roots, flowers and young leaves). *CrIDI1* produces both long and short transcripts giving rise to the corresponding proteins with and without a N-terminal transit peptide (TP), respectively (Guirimand *et al.*, 2012).

After IPP synthesis via the MEP pathway, a key step in the monoterpenoid branch towards the TIA pathway is the generation of geranyl diphosphate (GPP), the entry point to the formation of the monoterpene moiety, from the condensation of IPP and DMAPP by GPP synthase (GPPS) (Contin *et al.*, 1998; Hedhili *et al.*, 2007) (Fig. 2), which belongs to the family of short-chain prenyltransferases. In *C. roseus*, three genes were found to encode proteins with sequence similarity to the large subunit (CrGPPS.LSU) and the small subunit (CrGPPS.SSU) of heteromeric GPPSs, and a homomeric GPPSs, respectively (Rai *et al.*, 2013). CrGPPS.LSU is a bifunctional enzyme producing both GPP and geranyl geranyl diphosphate (GGPP). CrGPPS.SSU could function as a primary regulator to a certain extent in TIA biosynthesis, whereas CrGPPS is a homomeric enzyme forming GPP. Under normal conditions, both CrGPPS.LSU and heteromeric CrGPPS.LSU/CrGPPS.SSU provide a basal level of GPP for TIA formation in *C. roseus*. When under biotic/abiotic stress, the induced expression of SSU could cause an enhancement of GPPS activity by LSU and SSU interaction, thus increasing the GPP pool towards TIA biosynthesis (Rai *et al.*, 2013).

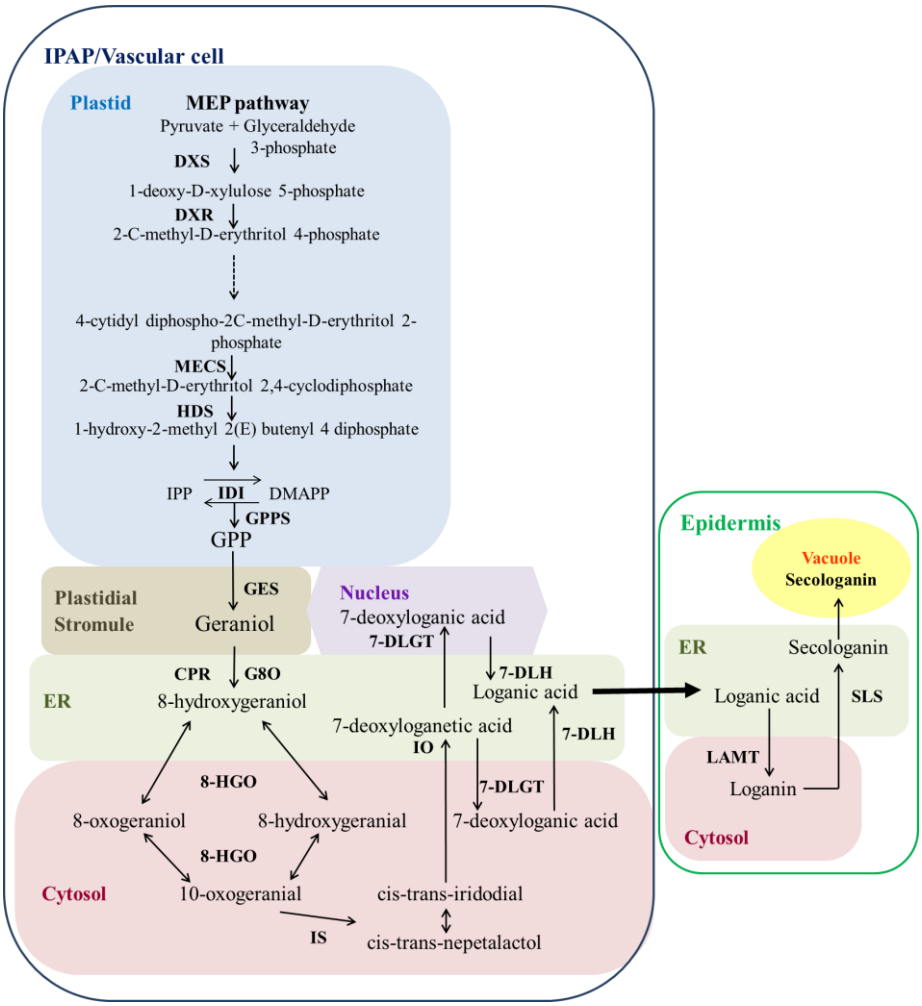

Fig. 2 The MEP-secoiridoid pathway and its compartmentation at intercellular and subcellular level. Solid arrows represent one-step reactions; broken arrows represent multiple or uncharacterized reactions.
IPAP, internal phloem-associated parenchyma; ER, endoplasmic reticulum.

The secoiridoid pathway starts with the formation of geraniol and ends with the production of secologanin (Fig. 2). Until 2011, only three steps (including the corresponding enzymes and genes) in the pathway were well established. One is the cytochrome P450 enzyme geraniol 10-hydroxylase

(G10H), also known as geraniol 8-oxidase (G8O, the name we will use here), responsible for the hydroxylation of geraniol into 8-hydroxylgeraniol (Collu *et al.*, 2001; Meijer *et al.*, 1993a), which also requires a cytochrome P450 reductase (CPR) for its functioning (Madyastha and Coscia, 1979; Meijer *et al.*, 1993b). The other two, are the last steps in the iridoid pathway, the enzymes loganic acid methyltransferase (LAMT) catalyzing methylation of loganic acid to form loganin (Murata *et al.*, 2008) and secologanin synthase (SLS), belonging to the cytochrome P450 (CYP) family, catalyzing the conversion of loganin to secologanin (Luijendijk *et al.*, 1998; Irmler *et al.*, 2000). Recently, the enzymes geraniol synthase (GES) (Simkin *et al.*, 2012), a member of the terpene synthase family, and iridoid synthase (IS), a cyclase recruited from short-chain oxidoreductase (Geu-Flores *et al.*, 2012), were cloned and their function in *C. roseus* was confirmed. The purified recombinant GES protein catalyzes the conversion of GPP into geraniol with a Km value of 58.5l M for GPP. The enzyme IS functions for the cyclization of 8-oxogeranial into iridodial, which probably couples an initial NAD(P)H-dependent reduction step via a Diels–Alder cycloaddition or a Michael addition (Geu-Flores et al., 2012). The enzymes for the remaining four steps were recently discovered and their encoding genes cloned (Asada *et al.*, 2013; Miettinen, 2013; Salim *et al.*, 2013). The enzyme after G8O, 8-hydroxygeraniol oxidoreductase (8-HGO) is able to oxidize the hydroxy group to an aldehyde in the substrates 8-hydroxygeraniol, 8-hydroxygeranial and 8-oxogeraniol in the presence of NAD+ to yield 8-hydroxygeranial, 8-oxogeraniol and 8-oxogeranial. The CYP enzyme iridoid oxidase (IO) catalyzes the conversion of iridodial into 7-deoxyloganetic acid, which is glucosylated by the enzyme7-deoxyloganetic acid glucosyltransferase (7-DLGT) forming 7-deoxyloganic acid using UDP-glucose as the sugar donor. Then the last step in the formation of the loganin skeleton is catalyzed by 7-deoxyloganic acid hydroxylase (7-DLH), which also belongs to the same P450 subfamily as SLS, yielding loganic acid (Miettinen, 2013). With these findings the secoiridoid pathway has now been fully characterized (Fig. 2). The elucidation clearly shows the evolution in biosynthesis elucidation. The first steps were identified by purification of the enzymes, sequencing part of the amino acid sequence and based on that probes where constructed for picking up the genes. This required knowledge of the intermediates of every single step in

a pathway, and the availability of these compounds as substrate for the enzyme assays. The lack of knowledge of the iridoid pathway was thus the major bottleneck in the past years. The recently developed fast sequencing methods opened new possibilities. For example, transcriptomic sequence data were obtained from *C. roseus* plants and cell cultures under different conditions. By comparing transcriptome, proteome and metabolome data from all these conditions, candidate genes could be selected and subsequently the encoded enzymes were overexpressed and tested for their putative activity, this approach was successful in the EU project Smartcell that aimed at elucidation of the total secoiridoid pathway (Dong *et al.*, 2013; Miettinen, 2013). Another approach is to silence the selected genes and through the analysis of the intermediates found in the plant the function can be deduced. This strategy was applied by DeLuca and co-workers who used a medicinal plants gene sequence database as basis (Asada *et al.*, 2013; Salim *et al.* 2013).

Besides the elucidation of the TIA pathway, its cellular and subcellular localization was also investigated (Fig. 2). The internal phloem associated parenchyma (IPAP) cells present in the periphery of stem pith or intraxylary on the upper part of the vascular bundles in leaves are the primary locations for the expression of genes in the MEP pathway (like *DXR*, *DXS*, *MECS*, *HDS, IDI*) (Mahroug *et al.*, 2007; Burlat *et al.*, 2004). RNA in situ hybridization showed that transcripts of *GES*, *IS*, *8-HGO*, *IO*, *7-DLGT* and *7-DLH* along with G8O are localized in the IPAPs like the genes involved in MEP pathway (Asada *et al.*, 2013; Miettinen, 2013; Geu-Flores *et al.*, 2012; Simkin *et al.*, 2012). That means that these cells produce loganic acid which is transported to e.g. the epidermis cell, where the loganic acid is methylated by the enzyme LAMT. The epidermis harbors also the maximum expression of the secologanine synthase (*SLS)* gene product, which produces the CYP72A1, catalyzing the last oxidation step in the iridoid pathway leading to secologanin (Irmler *et al.*, 2000; St-Pierre *et al.*, 1999; Yamamoto *et al.*, 2000). The Carborundum Abrassion (CA) approach led to the generation of a leaf epidermome-enriched cDNA library containing essentially all the known TIA pathway genes in *C. roseus* leaf epidermis (Murata *et al.*, 2008). Detailed analysis of this data set clearly revealed its abundance for genes like *SLS* and *LAMT* transcripts. From these findings it is concluded that both the MEP pathway and early steps of the

secoiridoid pathways take place in IPAP cells while the last two steps of the secoiridoid pathway are localized in the epidermis. Loganic acid is the mobile intermediate transferred from IPAPs to epidermis, which indicates that its transport might be a key biosynthetic rate controling point for the fluxes in secologanin production.

Subcellular compartmentation of the MEP-secoiridoid pathway was also sorted out. The MEP pathway occurs in plastids (Roytrakul and Verpoorte, 2007). Besides, the presence of HDS was also evidenced in long stroma-filled thylakoid-free extensions budding from plastids (Guirimand *et al.*, 2009). Expression of green fluorescent protein (GFP) fusions revealed that IDI is targeted to plastids, mitochondria and peroxisomes in *C. roseus* cells (Guirimand *et al.*, 2012). GFP localization indicated that CrGPPS.SSu is plastidial whereas CrGPPS is mitochondrial (Rai *et al.*, 2013). Transient transformation of *C. roseus* cells with a yellow fluorescent protein-fusion construct revealed that GES is localized in plastid stroma and stromules (Simkin *et al.*, 2012). G8O has now definitely shown to be localized in the endoplasmic reticulum (ER) (Guirimand *et al.*, 2009). IS was shown to be exclusively localized in the cytosol by using fluorescent protein fusions as well as bimolecular fluorescence complementation assays (Geu-Flores *et al.*, 2012). Using green fluorescent protein (GFP) fusions in *C. roseus* cells together with mCherry markers, IO and 7-DLH were revealed to be ER-associated, whereas 8-HGO and 7-DLGT are soluble proteins present in both cytosol and nucleus (Miettinen, 2013). LAMT forms homodimers in the cytosol, whereas SLS is anchored in the ER via an N-terminal helix (Guirimand *et al.*, 2011a). This fits the model of loganic acid being the transported intermediate that after uptake in the epidermis cells is methylated in the cytosol and subsequently further oxidized in the ER to yield secologanin. All these studies suggest a potential channel to export the MEP pathway product GPP from plastids, *via* stromules, to ER- and cytosol-anchored enzymes for iridoid biosynthesis.

The monoindole alkaloid biosynthesis and its localization

The biosynthesis of monoindole alkaloids starts from the central intermediate strictosidine, which is formed by a condensation reaction of tryptamine and secologanin catalyzed by strictosidine synthase (STR), from

where the diversion of metabolic fluxes starts towards different TIA biosynthetic pathways (De Waal *et al.*, 1995) (Fig. 3). In *C. roseus*, STR has at least 7 isoforms which are likely to be the products of posttranslational modifications, most likely through glycosylation as was shown for *Cinchona* STR (Stevens *et al.* 1993), since STR is encoded by a single gene (Mcknight *et al.*, 1990; Pasquali *et al.*, 1999). Strictosidine undergoes a deglucosylation reaction by strictosidine-β-D-glucosidase (SGD) enzyme to yield an unstable aglycon, cathenamine, which is a quite reactive carbinolamine and depending on the environment it can occur in different forms (Barleben *et al.*, 2007; Geerlings *et al.*, 2000; Lounasmaa and Hanhinen, 1998; Stöckigt *et al.*, 1977). Cathenamine is the branching point for three routes to different types of TIAs: 1) reduction to form ajmalicine by the enzyme cathenamine reductase (CR), which is further oxidized to serpentine by peroxidase in the vacuoles; 2) conversion of the iminium form of cathenamine into tetrahydroalstonine with cofactor NADPH by tetrahydroalstonine synthase (THAS), which finally is oxidized into alstonine; 3) reversible conversion to 4,21-dehydrogeissoschizine, which can be routed towards the formation of catharanthine and tabersonine/vindoline via stemmadenine. Neither enzymes nor genes involved in the catharanthine biosynthetic pathway have been identified so far (El-Sayed and Verpoorte, 2007), but a unique transporter for catharanthine was reported recently (see below, Yu and De Luca, 2013). On the other hand, the vindoline biosynthesis pathway beginning with tabersonine is quite well established. There are totally 6 enzyme-catalyzed steps in this pathway: hydroxylation of tabersonine by tabersonine 16-hydroxylase (T16H); O-methylation of 16-hydroxytabersonine by O-methyltransferase (16OMT); hydration of the 2,3-double bond of 16-methoxytabersonine by an unidentified hydroxylase; N(1)-methylation of 16-methoxy-2,3-dihydro-3-hydroxytabersonine by N-methyltransferase (NMT); hydroxylation at position 4 of desacetoxyvindoline by desacetoxyvindoline-4-hydroxylase (D4H); and the last step the 4-O-acetylation of deacetylvindoline by deacetylvindoline-4-O-acetyltransferase (DAT) to form vindoline. Except the yet unknown hydroxylase, for the other five enzymes the genes have been cloned and characterized. In roots, there are two alternative routes from tabersonine that instead of vindoline biosynthesis leads to other type of alkaloids (Verma *et al.*, 2012). One is the conversion into lochnericine

followed by the formation of hörhammericine, which is finally converted into 19-O-acetylhörhammericine by minovincinine-19-hydroxy-O-acetyltransferase (MAT). The other route starts with the formation of 6,7-dehydrominovincinine but the final product is also 19-O-acetylhörhammericine (Fig. 3).

In leaves, stems and flower buds, the epidermis cells harbor the *STR* mRNAs, whereas idioblast and laticifer cells embedded in the palisade tissue of the leaves represent the exclusive location of *D4H* and *DAT* mRNAs transcripts (St-Pierre *et al.*, 1999) (Fig. 3). In addition, *T16H* and *SGD* mRNAs were also preferentially present in the epidermis (Murata and De Luca, 2005; Murata *et al.*, 2008). High 16-OMT activity was detected in both abaxial and adaxial epidermal cell extracts, whereas the NMT activity is the highest in the whole leaf extract supporting its localization in the chloroplast thylakoids (Murata and De Luca, 2005; Murata *et al.*, 2008).

In the underground tissues, *STR* and *MAT* transcripts are localized in protoderm and cortical cells around the root apical meristem (Laflamme *et al.*, 2001; Moreno-Valenzuela *et al.*, 2003). The STR-GFP signal appeared in the vacuole in transient transformation experiments (Guirimand *et al.*, 2010a). The vacuolar targeting of STR is achieved via an ER-to-Golgi-to-vacuole route. SGD is targeted to the nucleus using a bipartite NLS and tends to multimerize in this cellular compartment (Guirimand *et al.*, 2010b). For the vindoline pathway, subcellular localization is fairly well understood. 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 the 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 the nucleocytoplasmic compartment because of their passive diffusion to nuclei due to their small protein size (Guirimand *et al.* 2011b).

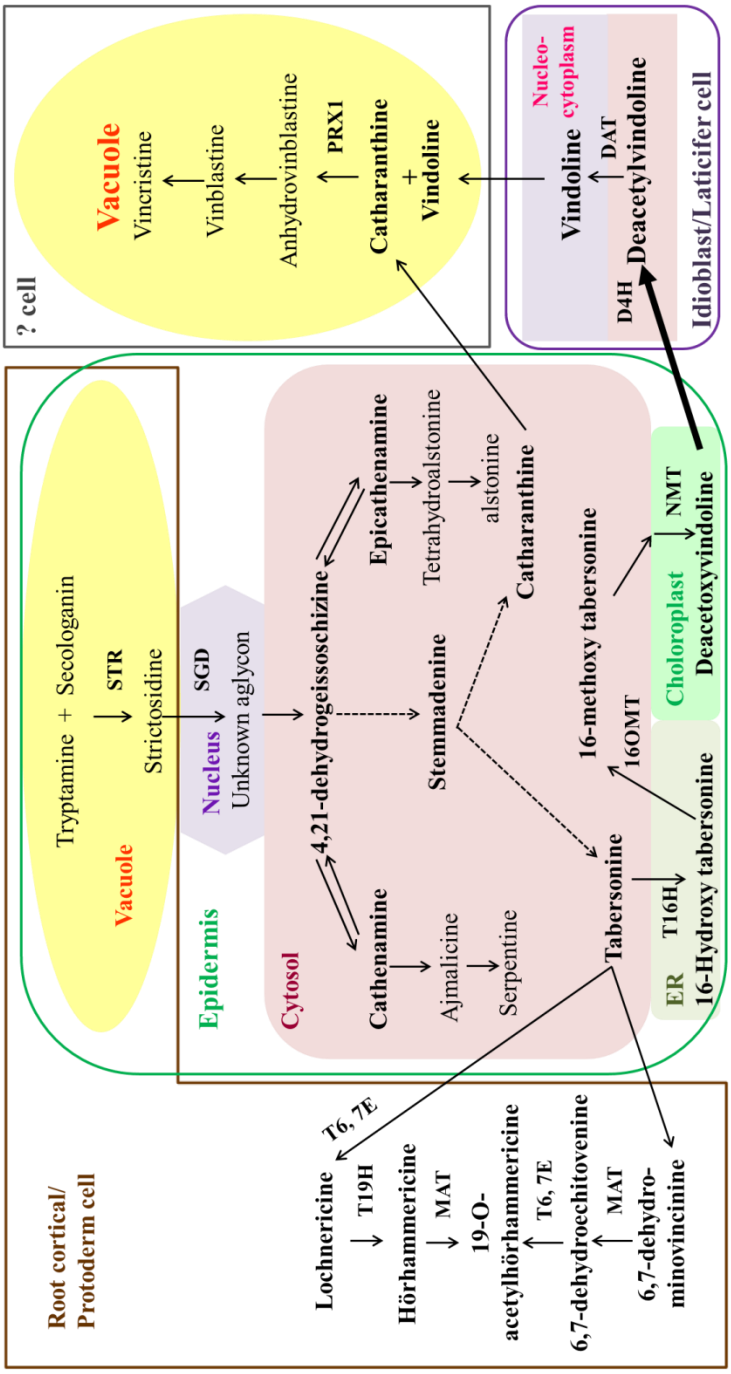

Fig. 3 The monoindole and bisindole alkaloids pathway and their compartmentation at intercellular and subcellular level. Solid arrows represent one-step reactions; broken arrows represent multiple or uncharacterized reactions.

The bisindole alkaloids and their localization

The first step of the bisindole alkaloid biosynthesis is the coupling of vindoline and catharanthine to produce anhydrovinblastine catalyzed by α-3',4'-anhydrovinbastine synthase (AVLBS) (Fig. 3), which is commonly known as CrPrx1 belonging to the class III basic peroxidases (Costa *et al.*, 2008). Anhydrovinblastine or its iminium ion is converted into vinblastine, which is finally converted into vincristine (Verpoorte *et al.*, 1997). Peroxidases are a family of isoenzymes found in all higher plants and are known to be involved in a broad range of physiological processes. In *C. roseus*, more novel peroxidase genes are cloned and characterized, such as CrPrx, CrPrx3 and CrPrx4 (Kumar *et al.*, 2007 and 2012). However, there is still limited knowledge about the enzymes and genes involved in the last two steps. The subcellular localization researches reported that *CrPrx1* is localized to vacuoles (Costa *et al.*, 2008), but *CrPrx* is apoplastic in nature (Kumar *et al.*, 2007).

Metabolic engineering of the TIA pathway in *Catharanthus roseus*

Metabolic engineering is an approach to modify metabolic pathways and metabolites production via gene transfer technology. With the increasing knowledge of the TIA pathway and its biosynthetic genes, metabolic engineering is widely performed on TIA biosynthesis in *C. roseus* to boost the yields of targeted TIAs. Different types of genes cloned from the TIA pathway have been overexpressed in the cells, hairy roots and plants of *C. roseus*, which showed different effects on TIA biosynthesis as well as on at the level of the full metabolome (Table 2).

Table 2 Overexpression of genes involved in TIA biosynthesis in the cell cultures, hairy roots and plants of *Catharanthus roseus*.

Varities	**Genes**	**Metabolites significantly affected in levels**	**References**
Hairy roots	*GmMYBZ2*	Catharanthine	Zhou *et al.* (2011)
	HMGR	Campesterol, serpentine, ajmalicine, catharanthine	Ayora-talavera *et al.* (2002)
	ASα	Tryptophan, tryptamine, lochnericine	Hughes *et al.* (2004)
	TDC	Serpentine	Hughes *et al.* (2004)
	ASα+ TDC	Tryptamine	
	ASα	Tryptamine	Hong *et al.* (2006)
	ASα +ASβ	Tryptophan, tryptamine	
	ASα +ASβ+TDC	Tryptamine	
	ASαβ	Naringin, catechin, salicylic acid	Chung *et al.* (2007)
	DAT	Hörhammericine	Magnotta *et al.* (2007)
	ORCA2	Catharanthine, vindoline	Liu *et al.*(2011)
	G8O(G10H)	Catharanthine	Wang *et al.* (2010)
	G8O+ORCA3	Catharanthine	
Cell cultures	*ORCA3*	Serpentine, ajmalicine, tabersonine, hörhammericine	Peebles *et al.* (2009)
	DXS	Ajmalicine, serpentine, lochnericine, tabersonine, hörhammericine	Peebles *et al.* (2011)
	Asα	Tryptophan, tryptamine, lochnericine, tabersonine, hörhammericine	
	DXS +G8O	Ajmalicine, tabersonine, lochnericine, hörhammericine	

	DXS+ Asα	tryptotamine, tabersonine, lochnericine, hörhammericine, tryptophan	Peebles *et al.* (2011)
	TDC	Tryptamine	Canel *et al.* (1998); Whitmer *et al.* (2002b)
	STR	Strictosidine, ajmalicine, catharanthine, serpentine, tabersonine	Canel *et al.* (1998); Whitmer *et al.* (2002a)
	PRX1	Ajmalicine, serpentine, H_2O_2	Jaqqi *et al.* (2011)
	ORCA3	Tryptophan, tryptamine	Van der Fits and Memelink (2000)
	CYP76B6	10-hydroxy geraniol	Collu *et al.* (2001)
	CjMDR1	Ajmalicine, tetrahydroalstonine	Pomahacova *et al.* (2009)
Plant	*DAT*	Vindoline	Wang *et al.*, (2012)

Structural genes

The expression of a more tryptophan inhibition resistant *Arabidopsis ASα* gene coupled with a glucocorticoid-inducible promoter in *C. roseus* hairy roots dramatically increased tryptophan and tryptamine yields but not of TIAs, except lochnericine, after induction with 3 μM dexamethasone (Hughes *et al.*, 2004a). Transgenic hairy roots expressing both *ASα* and *ASβ* subunits produced more tryptamine and showed a greater resistance to feedback inhibition of AS activity by tryptophan than those only expressing *ASα* (Hong *et al.*, 2006). When fed

with the terpenoid precursors 1-deoxy-D-xylulose, loganin, and secologanin respectively, hairy roots overexpressing *ASα* or *ASβ* could increase the levels of hörhammericine, catharanthine, ajmalicine, lochneriricine and tabersonine (Peebles *et al.*, 2006). As a side effect, the metabolic flux into the flavonoid pathway was also transiently increased when the *AS* overexpressing hairy roots were induced by 0.2 µM dexamethasone, which caused increases of catechin and naringin in hairy roots (Chung *et al.*, 2007). *TDC* overexpression in *C. roseus* transgenic calli results in increased tryptamine levels but not in increased TIA production (Goddijn *et al.* 1995), neither in *C. roseus* cell cultures (Whitmer *et al.*, 2002b). On the contrary, no increase of tryptamine but a 129% increase of serpentine was noted on induction of 3 µM dexamethasone in hairy roots overexpressing *TDC* (Hughes *et al.*, 2004b). Expressing *TDC* from *C. roseus* in cell cultures or plants of *Nicotiana tabacum* resulted in the formation of tryptamine up to 10 µg/g FW and 18 to 66 µg/g FW respectively (Hallard *et al.*, 1997). When co-overexpressing *AS* and *TDC* in hairy roots, they showed an enhanced ability to produce tryptamine, but only a transiently increased accumulation of tabersonine and lochnericine among all measured alkaloids (Hong *et al.*, 2006; Hughes *et al.*, 2004b). To study the effect of introducing TIA alkaloid biosynthetic genes in a plant normally only producing secologanin, Hallard and co-workers (Hallard, 2000; Geerlings *et al.*, 1999) introduced both the *TDC* and *STR* into *Cinchona* hairy roots. Compared to normal roots no more secologanin could be observed, whereas tryptamine, ajmalicine and serpentine could be detected in the hairy roots. This confirmed the presence of a glucosidase able to hydrolyze strictosidine. Though the alkaloids levels were very low, it shows that TIAs can also be made in non-alkaloid producing plants. That means alternative crops for making TIA. In that context also the production of strictosidine was achieved in yeast cells in which STR and SGD are overexpressed and which are fed with secologanin and tryptamine (Geerlings *et al.*, 2001). The cells could produce 3g/l of strictosidine in three days, many times more than ever achieved in cell cultures. As STR was mainly excreted to the medium, whereas SGD was in the cells, grinding the whole culture resulted in the production of cathenamine.

In the MEP pathway, the genes encoding 1-deoxy-D-xylulose-5-phosphate synthase (*DXS*), 1-deoxy-D-xylulose-5-phosphate reducto isomerase (*DXR*),

2-C-methyl-Derythritol-2,4-cyclodiphosphate synthase (*MECS*), hydroxymethylbutenyl-4-diphosphate (*HDS*) and isopentenyl diphosphate isomerase (*IDI1*) have been cloned and characterized from *C. roseus* (Chahed *et al.*, 2000; Veau *et al.*, 2000; Guirimand *et al.*, 2012). *DXS* overexpression resulted in a significant increase in ajmalicine, serpentine and lochnericine but a significant decrease in tabersonine and hörhammericine in *C. roseus* hairy roots. In fact, overexpression of *DXS* and *DXR* has been found to increase terpenoid production in several plants. For example, *DXS* overexpression enhances the production of various isoprenoids in *Arabidopsis* (Estevez *et al.*, 2001), and *DXR* overexpression increases essential oil yield in peppermint, carotenoid accumulation in ripening tomatoes (Mahmoud and Croteau, 2001; Rodriguez-Concepcion *et al.*, 2001), and various isoprenoids in tobacco leaves (Hasunuma *et al.*, 2008). There is no information about the use of *MECS* and *IDI1* in metabolic engineering on TIA production.

At the edge of primary and secondary metabolism, G8O as gatekeeper could be a carbon flux controlling step for the iridoid pathway. The encoding gene was overexpressed in the hairy roots of *C. roseus*, which resulted in a higher accumulation of catharanthine (0.063–0.107% on a dry weight) than in the wild-type lines (0.019 and 0.029%) (Wang *et al.*, 2010). When co-overexpressing *DXS* and *G8O*, the hairy roots showed a significant increase in ajmalicine by 16%, lochnericine by 31% and tabersonine by 13% (Peebles *et al.*, 2010). Considering their location with respect to the IPP/DMAPP branching point for terpenoid classess, it is conceivable that the overexpression of one downstream structural gene alone is unable to effect the channeling of the flux at this upstream branch point while the overexpression together with an upstream gene of the IPP/DMAPP branch point may affect the carbon fluxes by a push and pull effect toward the TIA iridoid precursor. An increased production of IPP/DMAPP and *G8O* overexpression may increase the flux in the monoterpenoid branch and from there into TIA. *DXS* and *ASα* co-overexpression displayed a significant increase in hörhammericine by 30%, lochnericine by 27% and tabersonine by 34% in hairy roots (Peebless *et al.*, 2010). Recent discoveries of IDI1, GPPS, GES and iridoid synthase encoding genes provide new possibilities for the regulation and improvement of TIA production.

Cultures of *STR* transgenic cells consistently showed ten-fold higher STR activity than wild-type cultures, which favored the biosynthetic flow through the pathway. Two such lines accumulated over 200 mg/L of strictosidine and/or strictosidine-derived TIAs, including ajmalicine, catharanthine, serpentine, and tabersonine, while maintaining wild-type levels of TDC activity (Canel *et al.*, 1998). Whitmer *et al.* (2002a,b) showed that in *C. roseus* cell lines overexpressing *TDC* or *STR* had an overcapacity in indole alkaloid biosynthesis enzyme activity, as feeding of loganin resulted in a large increase of alkaloid production whereas the combination of loganin and tryptamine feeding even further increased the level of alkaloids. Apparently the iridoid pathway is the most limiting step, but when that limitation is overcome the tryptophan pathway becomes limiting. Overcoming one limting step immediately shows what the next limiting step is. A single structural gene overexpression will thus always have only a limited effect on the overall flux in a pathway. On the other hand it shows that probably many biosynthetic steps are already present, and only the enzymatic machinery has to be started up by increasing the amount of the limiting substrate by feeding or genetic modification. The elucidation of the full iridoid pathway as described above is thus a major breakthrough opening new possibilities to explore for increasing TIA production.

The key gene *DAT* for the vindoline biosynthesis was introduced into *C. roseus* plants by *Agrobacterium tumefaciens*, which resulted in an increase of vindoline level in the leaves (Wang *et al.*, 2012). However, overexpression of *DAT* in hairy roots altered their TIA profile and accumulated more hörhammericine compared to control lines (Magnotta *et al.*, 2007). Comparative analysis revealed that TIA pathway genes have elevated expression levels in *CrPrx* overexpression transgenic hairy roots, whereas they had a significant reduction in their transcript level in *CrPrx-RNAi* transgenic hairy roots (Jaggi *et al.*, 2011). Alkaloid analysis showed higher levels of ajmalicine and serpentine in these peroxidase overexpressing cell lines. All these transgenic lines produced higher amounts of H_2O_2 (Jaggi *et al.*, 2011). The oxidative burst or H_2O_2 production is closely related to indole alkaloid production (Zhao *et al.*, 2001). In leaves of *C. roseus*, PRX together with phenolic compounds was suggested to represent an important sink/buffer of excess H_2O_2, diffusing from the chloroplast under high light exposure (Ferreres *et al.*, 2011). These results

indicate a role of the *CrPrx* gene in the regulation of TIA pathway and other metabolic pathways, thus affecting the production of specific alkaloids. In order to study the role of CrPrx and CrPrx1 in plants, these two peroxidases were expressed in *Nicotiana tabacum* (Kumar *et al.,* 2012). The transformed plants exhibited increased peroxidase activity. Increased oxidative stress tolerance was also observed in transgenics when treated with H_2O_2 under strong light conditions. However, differential tolerance to salt and dehydration stress was observed during germination of T1 transgenic seeds. Under these forms of stress, the seed germination of *CrPrx*-transformed plants and wild-type plants was clearly suppressed, whereas *CrPrx1* transgenic lines showed improved germination. *CrPrx*-transformed lines exhibited better cold tolerance than *CrPrx1*-transformed lines. These results indicate that vacuolar peroxidases play an important role in salt and dehydration stress, while cell wall-targeted peroxidases render cold stress tolerance.

Transporter genes

Since TIA biosynthesis involves at least 4 different cell types and in each of them at least 5 different subcellular compartments, the trafficking of pathway intermediates from one to another compartment requires an efficient transport system. Previous research also suggested that transport is one of the potential factors in regulation of TIA biosynthesis. However, the knowledge about TIA membrane transport mechanisms is still very limited.

Transport has basically two aspects, a physicochemical and a biochemical one. In cells and in an organism diffusion will always take place. Concentration gradient driven molecules diffuse in a liquid phase, and they will move from an aqueous phase to lipid phase (membrane). Mass transfer factors determine the rate of the uptake in a lipophilic membrane from water and the release again to water, i.e. the transport rate through a membrane. That allows calculations of the rate of diffusion of compounds between cells and cellular compartments. The complexity of this system is further increased by the pH, making that acids and bases at different pH have different solubility in the liquid phases. For example, an alkaloid in acidic conditions is poorly soluble in a lipid phase, but at basic conditions it is better lipid soluble. So at a high ratio of protonated to non-protonated alkaloids, which is at acidic conditions transport, will be slow

through a membrane, at higher pH it will be the opposite. Modeling uptake in *C. roseus* vacuoles using these physical-chemical processes resulted in an ion-trap model for alkaloid uptake in vacuoles that fitted reasonably well the experimental results using isolated vacuoles. The lower pH in the vacuole than in the cytosol causes preferred accumulation of alkaloids in the vacuole if compared with the cytosol as the uptake rate on the more basic cytosolic site of the membrane is faster than on the more acidic vacuolar side. This physicochemical process requires ATP for maintaining the low vacuolar pH, so depletion of ATP will inhibit uptake, similar as in case of ABC transporters (Blom *et al.*, 1991). On the other hand Deus-Neumann and Zenk (1984) reported uptake kinetics for active transport for some indole alkaloids. Ajmalicine, catharanthine and vindoline showed different rates, and all were ATP dependent. From this it was hypothesized that the vacuolar transport occurred via selective transporter proteins. Roytrakul (2004) reported a detailed study on the uptake of several *C. roseus* alkaloids and secologanin in isolated vacuoles. By adding inhibitors of the various classes of transport proteins, for each individual compound quite a different and complex picture came out. For each single compound different transporter seem to be involved (Roytrakul and Verpoorte, 2007).

The uptake into the vacuole is thus dependent on a combination of factors, first of all there is the bidirectional diffusion driven transport. On top of that there are Multi Drug Resistant associated Proteins (MRP, inhibited by glibenclamide) and ATP Binding Cassette (ABC, inhibited by *ortho*-vanadate) type of transporters involved in uptake. Whereas Multidrug resistant (MDR) (P-glycoproteins, inhibited by cyclosporine A and verapamil) and MDR coupled with proton symport, are responsible for extrusion. To further complicate the transport system, glutathione was found to cis-activate the MRP transport of ajmalicine into the vacuole (Roytrakul 2004, Roytrakul and Verpoorte, 2007). Considering the multicompartment system involved in the TIA biosynthesis, it is clear that with the already very complex transport system into vacuoles, the model for a single-cell or multi-cell system is impossible to describe. The need for sufficient energy and co-factors in the different compartment add further to this complexity. In an attempt to calculate the rate of transport between cells by using the various available data on uptake of

compounds and a number of assumptions based on observations from other plants, it became clear that at least diffusion alone would result in a biosynthetic rate more or less of what is found in the plant (Supandi *et al.*, 2009, unpublished results). That means that the selective transport might play a role in some of the specific biosynthetic steps, e.g. by accumulating certain compounds in a vacuole, where they are oxidized to yield serpentine or dimeric alkaloids. In case of serpentine, this anhydronium compound is much more polar than ajmalicine from which it is formed by oxidation, thus becomes trapped into the vacuole. The fact that tobacco vacuoles excrete strictosidine, whereas *C. roseus* vacuoles store it (Hallard *et al.*, 1997) shows at least that every plant species will have different transport systems with different selectivity. Considering that the TIAs are confined to certain cell types may also in part be due to specific transport systems in the cellular membrane(s). That means that introduction of a novel pathway in a plant maybe hampered by lack of transport of intermediates.

The example of *CjMDR1*, an ABC transporter gene specific for berberine transport originally isolated from *Coptis japonica*, shows the problems one may encounter in genetically modifying transport. This gene was expressed in *C. roseus* cell cultures (Pomahacova *et al.*, 2009). The endogenous alkaloids ajmalicine and tetrahydroalstonine were accumulated significantly more in *C. roseus* cells expressing *CjMDR1* in comparison with control lines after feeding these alkaloids, but transport of other alkaloids was not affected, and even no effect at all on berberine transport into the cells was observed.

A unique catharanthine ABC-transporter (*CrTPT2*) belonging to the pleiotropic drug resistance (PDR) family has been cloned and functionally characterized. It is expressed predominantly in the epidermis of young leaves (Yu and De Luca, 2013). Further analysis suggested that CrTPT2 may be specific to TIA-producing plant species, where it mediates secretion of alkaloids to the leaf surface. *CrTPT2* gene expression is induced under the treatment with catharanthine, and its silencing redistributes catharanthine into the leave, causing an increase of dimeric alkaloids levels in the leaves.

Recently strong support for active TIAs uptake by *C. roseus* mesophyll vacuoles through a specific H (+) antiport system was reported (Carqueijeiro *et al.*, 2013). The vacuolar transport mechanism of the main TIAs accumulated in *C. roseus* leaves, vindoline, catharanthine, and α-3',4'-anhydrovinblastine, was

characterized using a tonoplast vesicle system. Vindoline uptake was ATP dependent, and this transport activity was strongly inhibited by NH_4^+ and carbonyl cyanide m-chlorophenyl hydrazine and was insensitive to the ATP-binding cassette (ABC) transporter inhibitor vanadate. Spectrofluorimetric assays with a pH-sensitive fluorescent probe showed that vindoline and other TIAs indeed were able to dissipate an H^+ preestablished gradient across the tonoplast by either vacuolar H^+-ATPase or vacuolar H^+-diphosphatase. Though it was claimed that this system would be responsible for the TIA transport instead of an ion-trap mechanism or ABC transporters, it seems unlikely, as at least physicochemical based transport will always occur and the various previous reports found alkaloid specificity for the uptake into the vacuole.

Transcription factors

Transcription factors (TFs) are sequence-specific-DNA-binding proteins that interact with the promoter regions of target genes and modulate the rate of mRNA synthesis by RNA polymerase II (Gantet and Memelink, 2002). They usually control the expression of more than one gene vital for normal development and functional physiology in plants. Several TFs have been found to be involved in the regulation of secondary metabolism. In *C. roseus*, TIA biosynthesis is related with plant defense and controlled by a number of signals including developmental cues, light, and biotic and abiotic stress. Regulation of TIA biosynthetic genes is coordinated by several types of TFs.

The best-known TFs regulating TIA biosynthesis are the jasmonates-responsive ORCAs (octadecanoid-responsive *Catharanthus* AP2-domain proteins) from the plant-specific AP2/ERF (APETALA2/ethylene-responsive factor) family, i.e. ORCA2 and ORCA3, for which the regulation mechanism of the TIA biosynthetic genes in *C. roseus* is well established. *ORCAs* expression is induced by jasmonates (van der Fits and Memelink, 2001), which is a major and essential signaling pathway to induce TIA biosynthesis. Jasmonates are first converted to the bioactive jasmonate isoleucine derivative (JA-Ile). Perception of JA-Ile by CrCOl1 causes the degradation of the CrJAZ proteins, derepressing the CrMYC2 protein. CrMYC2 then activates the expression of *ORCAs*, which in its turn activate the expression of TIA biosynthetic genes through binding to the JERE (jasmonate

and elicitor-responsive element) in the promoter of targeted genes (Menke *et al.*, 1999; van der Fits and Memelink, 2000; Zhang *et al.*, 2011). Ectopic expression of *ORCA3* in cell cultures of *C. roseus* increased the expression of the TIA biosynthetic genes *TDC*, *STR*, *CPR* and *D4H*, as well as two genes encoding primary metabolic enzymes (*AS* and *DXS*) (van der Fits and Memelink, 2000). This indicates that ORCA3 is a central regulator of TIA biosynthesis and positively regulates the biosynthesis of TIAs and their precursors. Nevertheless, ORCA3 does not regulate the expression of *G8O* and *DAT*. Overexpression of *ORCA3* caused an increase of ajmalicine and serpentine but a decrease in tabersonine, lochnericine, and hörhammericine in hairy roots (Peebles *et al.*, 2009). When *ORCA3* combined with *G8O* were overexpressed in hairy roots, alkaloid accumulation level analyses showed that all transgenic clones accumulated more catharanthine, with the highest accumulation level 6.5-fold more than that of the non-expression clone (Wang *et al.*, 2010). ORCA2 from *C. roseus* was demonstrated to regulate the expressions of *STR*, *TDC* and *G8O* gene, but has no effect on the CYP related reductase (*CPR*), which is regulated by ORCA 3 (Li *et al.*, 2013; Menke *et al.*, 1999). Transgenic hairy root cultures overexpressing *ORCA2* showed an average content of catharanthine that was increased up to 2.03 in comparison to the control lines, respectively. However, vinblastine could not be detected in the transgenic and control hairy root cultures by HPLC (Liu *et al.*, 2011).

The zinc finger-binding proteins ZCT1, ZCT2, and ZCT3 (members of the transcription factor IIIA-type zinc finger family) were found to bind to the promoters of *STR* and *TDC*. This interaction repressed the activity of *STR* and *TDC*. The binding of the ZCTs to the *STR* promoter has been suggested to counteract the activation of *STR* by ORCA2 or ORCA3 (Pauw *et al.*, 2004).

Using an enhancer domain of the *STR* promoter as bait in a yeast one-hybrid screen resulted in the isolation of *CrBPF1*, a periwinkle homolog of the MYB-like transcription-factor BPF1 from parsley (van der Fits *et al.*, 2000). *CrBPF1* expression is induced by elicitors but not jasmonates, which indicates that elicitors induce *STR* expression in periwinkle cells via jasmonic-acid-dependent and -independent pathways.

Sequence analysis of the *STR* and *TDC* promoters shows that they contain a G-box or G-box-like binding site. Two G-box-binding factors, CrGBF1 and

CrGBF2, were subsequently identified in *C. roseus* and shown to repress the transcription of *STR* by binding to the G-box sequence (Siberil *et al.*, 2001).

A *C. roseus* WRKY transcription factor, CrWRKY1, is preferentially expressed in roots and induced by the phytohormones jasmonate, gibberellic acid and ethylene (Suttipantaa *et al.*, 2011). Overexpression of *CrWRKY1* in *C. roseus* hairy roots upregulated several key TIA pathway genes, especially *TDC*, as well as transcriptional repressors *ZCT1*, *ZCT2* and *ZCT3*. However, *CrWRKY1* overexpression repressed the transcriptional activators, *ORCA2*, *ORCA3* and *CrMYC2*. Overexpression of a dominant repressive form of CrWRKY1, created by fusing the SRDX-repressor domain to CrWRKY1, resulted in down-regulation of *TDC* and *ZCTs* but up-regulation of *ORCA3* and *CrMYC2*. CrWRKY1 binds to the W-box elements of the *TDC* promoter in the electrophoretic mobility shift, yeast one-hybrid and *C. roseus* protoplast assays. Up-regulation of *TDC* increased TDC activity, tryptamine concentration and resistance to 4-methyl tryptophan inhibition of *CrWRKY1* hairy roots. Compared to control roots, *CrWRKY1* hairy roots accumulated up to 3-fold higher levels of serpentine. The preferential expression of *CrWRKY1* in roots and its interaction with transcription factors including ORCA3, CrMYC2 and ZCTs may play a key role in determining the root-specific accumulation of serpentine in *C. roseus* plants.

The root-specific MADS-box transcription factor Agamous-like 12 (Agl12) from *Arabidopsis thaliana* was expressed on the differentiation of suspension cells from *C. roseus* (Montiel *et al.*, 2007). The expression of Agl12 is sufficient to promote an organization of suspension cells into globular parenchyma-like aggregates but is insufficient by itself to induce complete morphological root differentiation. Agl12 expression selectively increases the expression of genes encoding enzymes involved in the early biosynthetic steps of the terpenoid precursor of the alkaloids. The transgenic cell lines expressing Agl12 produced significant amounts of ajmalicine, which indicates that TFs involved in tissue or organ differentiation may constitute new metabolic engineering tools to produce specific valuable TIAs. Murata and De Luca (2005) reported that ORCA3 and an AP2/ERF type of transcription factors were expressed in all four cell types (epidermis, IPAP, laticifers and idioblast cells).

NMR-based Metabolomics *Catharanthus roseus*

As TIA biosynthesis is such a complex system, multiple techniques are required to elucidate the pathways and its regulation thereof. In the postgenomic era, metabolomics is the latest tool for functional genomics (Sumner *et al.*, 2003). Metabolomics is a fast growing powerful technology and is useful for phenotyping and diagnostic analyses of plants (Schauer and Fernie, 2006). It is rapidly becoming a key tool in functional annotation of genes and in the comprehensive understanding of the cellular response to various biological conditions. Metabolomic approaches have recently been used to assess the natural variance in metabolite content between individual plants, an approach with great potential for the improvement of the compositional quality of crops. Metabolomics covers metabolic profiling, fingerprinting, footprinting and metabolic flux analysis. Metabolomics can be used to measure the effect of developmental stage, environment, the daily and seasonal changes in the plant metabolome; the metabolic response to stress, and chemotaxonomy, which eventually can be used in identification of the genes involved as for example shown for the elucidation of the iridoid pathway in *C. roseus* (Miettinen, 2013). Metabolomics in combination with transcriptomics thus became a major tool of functional genomics. Moreover, metabolomics is becoming an important tool in the quality control of food and medicinal plants, as well as a diagnostic tool in health care.

Nuclear magnetic resonance (NMR) is a very powerful method that allows the simultaneous detection of diverse groups of secondary metabolites (flavonoids, alkaloids, terpenoids, etc.) besides the abundant primary metabolites (sugars, organic acids, amino acids, etc.) (Kim *et al.*, 2010). The non-selectiveness of NMR makes it an ideal tool for unbiased plant metabolomics studies. As signals are proportional to their molar concentration in an NMR spectrum, it is possible to make the direct comparison of concentrations of all compounds without the need for calibration curves of each individual compound. Moreover, the time of analysis is short and the sample preparation is simple and fast, enabling the analysis of large numbers of samples per hour, without the need for calibration curves for all single compounds for quantitation. These are major advantages if compared with the

more sensitive methods as MS, LC-MS and GC-MS, but NMR suffer from lack of absolute quantitative data. It is thus a choice between the quality of data: a large number of metabolites with only relative quantitation for each compound, or smaller number of metabolites with full quantitation. However, one should keep in mind that in all present metabolomics methods, the visible metabolome is determined, or may be better to say limited, by the method of extraction! Only soluble compounds will be visible, and poorly soluble compounds will always be present up to saturation levels, thus not showing any variation above that level, even when large differences in accumulation may occur in the plant. As the first choice for metabolomics, NMR-spectroscopy has been applied to plant metabolomic studies of *C. roseus* in every aspect, i.e. identification of novel metabolites, elucidation of metabolic pathways, metabolic responses to stress, metabolic characterization and classification, and metabolic flux analysis. NMR is also a very useful technique for structure elucidation using various 2D NMR measurements without further fractionation of the extract. Thus dozens of primary and secondary metabolites were identified in cell cultures, hairy roots, and plants of *C. roseus*.

Metabolic profiling/fingerprinting combined with multivariate data analysis

Metabolite profiling, fingerprinting and footprinting are commonly used as efficient methods in metabolomics studies. Profiling aims at quantitative analysis of sets of metabolites in a selected biochemical pathway or a specific class of compounds. Fingerprinting uses high throughput qualitative screening of the metabolic composition in an organism or tissue with the primary aim of sample comparison and discrimination analysis. Footprinting is the fingerprinting analysis of metabolites that are excreted by cells to the culture medium. Multivariate or pattern recognition techniques such as the well-described principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA) and hierarchical cluster analysis are useful tools to analyze complex data sets (Summer *et al.*, 2003). PCA and PLS-DA are the two widely applied regression methods used to reduce the multidimensionality of the metabolomics data. They provide an excellent

platform to study, for example, the stress response in plants; quality control and authentication of medicinal plants (like *Artemisia annua*, *Angelica acutiloba*, and *Panax notoginseng*); classification of different plant species genotypes or ecotypes; identification of biomarkers for disease diagnosis; or identification of bioactive compounds in plants. In fact metabolomics has become the tool for systems biology, studying the response of the whole system, rather than a reductionist approach in which only a few parameters are measured. At the same time there is also the question what is the metabolome of a plant, in fact it is the mixture of the metabolome of different tissues and even cells. One may thus speak about a macrometabolome and a micrometabolome, and even nanometabolome if one regards the role of cellular compartments in the cellular metabolism. The above discussed localization of the TIA biosynthesis over different cells and cellular compartments shows that for a better understanding of the biosynthesis a single cell or at least single cell type metabolomics would be of great value. First experiments in that direction have already been made. The analysis of epidermis cells is a clear example (Murata and De Luca, 2005; Murata *et al.* 2008).

Inner and outer cells of *C. roseus* calli treated with various elicitors in solid-state cultures were differentiated by ^{1}H NMR spectrometry and PCA (Yang *et al.*, 2009). The cells with different localization in the calli treated with different elicitors and relative locations could be separated in the PCA score plots. Especially, there was a clear separation between non-treated samples and those co-treated with silver nitrate and MeJA.

To understand stress response in *C. roseus*, a comprehensive metabolomic profiling of the leaves infected by 10 types of phytoplasmas was carried out using one-dimensional and two-dimensional NMR spectroscopy followed by PCA (Choi *et al.*, 2004). The results showed that major discriminating factors to characterize the phytoplasma-infected *C. roseus* leaves from healthy ones were increases of metabolites related to the biosynthetic pathways of phenylpropanoids and terpenoid indole alkaloids: chlorogenic acid, loganic acid, secologanin, and vindoline. Furthermore, higher abundance of glycine, glucose, polyphenols, succinic acid, and sucrose were detected in the phytoplasma-infected leaves. Based on the NMR and PCA analysis, it seems that the biosynthetic pathway of terpenoid indole alkaloids, together with that of

phenylpropanoids, is stimulated by the infection with a phytoplasma. The effect of salicylic acid (SA) on the metabolic profile of *C. roseus* cell cultures in a time course (0, 6, 12, 24, 48 and 72 h after treatment) was studied using ^{1}H-NMR spectrometry and PCA (Mustafa *et al.*, 2009a). Adding 25 μmol of sodium SA into 100 mL of 5 days-old cell cultures altered the metabolome compared with the non-treated cells. A dynamic change in amino acids, phenylpropanoids, and tryptamine was found in cells at 48 h after SA treatment. Additionally, 2,5-dihydroxybenzoic-5-*O*-glucoside was detected only in SA-treated cells (Mustafa *et al.*, 2009a).

Metabolic flux analysis based on ^{13}C labeling experiment

One of the problems of metabolomics is that it is like a picture, it measures the amounts of compounds present at a certain time point, but it does not tell anything about the turnover. A major compound can be a stored product or part of a very active metabolic pathway, only measuring the dynamics of the system can give the answer, that means measuring the flux through pathways, making a film, rather than a picture.

Metabolic flux analysis (MFA), the quantification of all intracellular fluxes in an organism, is thus an important cornerstone of metabolic engineering and systems biology. Each flux reflects the function of a specific pathway within the network. As all biological activity is related to metabolic activity, it is these fluxes that deliver the phenotype of an organism (Ratcliffe and Shachar-Hill, 2005). Flux measurements complement transcriptomic, proteomic, and metabolomic technologies in defining phenotypes, and provide a useful complementary parameter for the system-wide characterization of metabolic networks. MFA on different phenotypes in plants can provide valuable information, which facilitates to select metabolic engineering targets, elucidate metabolic pathways, and construct metabolic models (Stephanopoulos and Stafford, 2002). Metabolic flux analysis is usually carried out using ^{13}C-NMR and ^{1}H-^{13}C HSQC NMR analysis, GC-MS or LC-MS in experiments where ^{13}C, ^{2}H or ^{15}N isotope-labeled compounds are added to the organisms studied. The chromatographic-MS approaches do allow to determine the overall percentage of labeling, but not like in NMR, the site of incorporation. The data are analysed and interpreted by mathematical models and software like ^{13}C-FLUX^{TM} and 4F

(Ratcliffe and Shachar-Hill, 2005). The ^{13}C isotope is widely used since it is not radio-active and NMR analysis allows determining the precise site of the label in a molecule. Natural abundance of ^{13}C is 1.1%, so already a labeling of 1.1% will lead to a doubling of the percentage of the carbon being labeled and consequently to a clear increase of the signal concerned. ^{13}C labeling experiments (^{13}CLE)-based MFA have been applied to *C. roseus* for pathway elucidation, for finding crosslinks between pathways, and for flux quantification in the central carbon metabolism.

In *C. roseus*, mevalonate was first considered to be the exclusive precursor of isopentenyl diphosphate in the biosynthesis of secologanin. However, later research indicated that the MEP pathway might be the alternative pathway involved. A feeding experiment using [1-^{13}C] glucose to *C. roseus* cell cultures followed by analysis of its incorporation into secologanin using ^{13}C NMR spectroscopy was performed. The data on the sites of incorporation of the ^{13}C label showed that the MEP pathway and not the mevalonate pathway was the major route for secologanin biosynthesis (Contin *et al.*, 1998). The biosynthetic pathways of SA and 2,3-dihydroxybenzoic acid (2,3-DHBA) were studied through a similar feeding-NMR method. The data led to the conclusion that the isochorismate pathway is responsible for the biosynthesis of both compounds, presenting the first full chemical evidence for the isochorismate pathway for the biosynthesis of SA as an important signal molecule plants (Mustafa *et al.*, 2009b; Budi Muljono *et al.*, 2002a,b).

In order to assess quantitatively the crosstalk between the MEP pathway and the mevalonate pathway, [2-$^{13}C_1$] mevalonolactone or [U-$^{13}C_6$] glucose were supplied to *C. roseus* cell cultures grown in light or dark (Schuhr *et al.*, 2003). The incorporations of exogenous [2-$^{13}C_1$] mevalonolactone were 48% and 7% into the DMAPP and IPP precursors of sitosterol and lutein, respectively. With [U-$^{13}C_6$] glucose as precursor, at least 95% of sitosterol precursors were obtained from the mevalonate pathway, whereas phytol appeared to be biosynthesized via the deoxyxylulose phosphate pathway (approximately 60%) as well via the mevalonate pathway (approximately 40%).

Hairy roots of *C. roseus*, as a pharmaceutically significant plant compounds production system and an important metabolic engineering target, were used as a model system in the study of CLE-based MFA. [U-$^{13}C_6$] glucose

was fed to the hairy roots of *C. roseus* to investigate its elemental and biomolecular composition, in which the abundances of lipids, lignin, cellulose, hemicellulose, starch, protein, proteinogenic amino acids, mineral ash, and moisture were quantified (Sriram *et al.*, 2006). Moreover, 12 biomass synthetic fluxes were precisely calculated in the metabolic map of the plant system of *C. roseus* hairy roots. The results show the flux of the carbons from β-glucose consumed by the hairy roots into various products, which enables the design of metabolic engineering strategies to divert carbon to the economically attractive TIAs (Sriram *et al.*, 2006). The application of "bondomers", which are isomers of a metabolite differing in the connectivity of their C-C bonds, was introduced to MFA study as a computationally alternative to the isotopomer concept in *C. roseus* (Sriram *et al.*, 2007). Hairy roots were cultured on (5% w/w [U–^{13}C6], 95% w/w naturally abundant) sucrose. HSQC and COSY spectra of the hydrolyzed aqueous extract was acquired from the hairy roots. Analysis of these spectra yielded a data set of 116 bondomers of beta glucans and proteinogenic amino acids from the hairy roots. Fluxes were evaluated from the bondomer data by using comprehensive bondomer balancing, most of which were identified in a three-compartment model of central carbon metabolism with good precision. Pentose phosphate pathways were observed to occur in parallel in the cytosol and plastids with significantly different fluxes. The fluxes between phosphoenolpyruvate and oxaloacetate in the cytosol and between malate and pyruvate in the mitochondria were relatively high (60.1 ± 2.5 mol per 100 mol sucrose uptake, or 22.5 ± 0.5 mol per 100 mol mitochondrial pyruvate dehydrogenase flux).

The development of a comprehensive flux analysis tool for the plant system of *C. roseus* is expected to be valuable in assessing the metabolic impact of genetic or environmental changes.

Combination of omics tools for system biology

Integration with multiple omics is exercised to predict gene functions and characterize the complex interaction and coordination of plant metabolic network in biological processes from a system biological point of view (Fukushima *et al.*, 2009b). Combination of non-targeted approaches, such as transcriptomics and metabolomics, can reveal potential gene-to-metabolite

networks (Urbanczyk-Wochniak *et al.*, 2003), filter out candidate genes for certain metabolic pathways (Okazaki *et al.*, 2009), and suggest gene functions by overexpression (Lackman *et al.*, 2011). The integration of omics approaches can help to reveal the organization of the whole system and thus to identify interesting targets for further studies. A good exampe is the study on the circadian clock mechanism of *Arabidopsis thaliana* (Fukushima *et al.*, 2009a). In total seven mutants were investigated the physiological relevance of Pseudo Response Regulators (PRR 9, 7, and 5) in *Arabidopsis*, including two arrhythmic plants, a *Cicardian Clock-Associated1* overexpressor line (*CCA1*-ox) and a *PRR 9, 7,* and *5* triple mutant (*d975*). Based on metabolite profiling, *d975* displayed a dramatic increase in intermediates in the tricarboxylic acid cycle while *CCA1*-ox showed less change in primary metabolism. The integrated approach with transcriptomics and metabolomics data showed that PRR9, 7, and 5 downregulate the biosynthetic pathways of chlorophyll, carotenoids and abscisic acid, and α-tocopherol, highlighting them as additional outputs of pseudo-response regulators. These results suggested that mitochondrial functions are coupled with the circadian system in plants (Fukushima *et al.*, 2009a).

A comprehensive profiling analysis of *C. roseus* was performed by combining genome-wide transcript profiling of cDNA-amplified fragment-length polymorphism with metabolic profiling of elicited *C. roseus* cell cultures to yield a collection of known and previously undescribed transcript tags and metabolites associated with TIAs (Rischer *et al.*, 2006). Previously undescribed gene-to-gene and gene-to-metabolite networks were drawn up by searching for correlations between the expression profiles of 417 gene tags and the accumulation profiles of 178 metabolite peaks. These networks revealed that the different branches of terpenoid indole alkaloid biosynthesis and various other metabolic pathways are subject to different hormonal regulation. These networks also served to identify a select number of genes and metabolites likely to be involved in the biosynthesis of terpenoid indole alkaloids. So, the combination of multiple omics tools should contribute greatly to identification of key regulatory steps and characterization of the pathway interaction in various processes, aiming at elucidating the systemic coordination and communication among plant metabolic network.

The future prospects

With the rapid development of novel tools of modern plant molecular biology and chemistry, the knowledge of TIA biosynthesis and its regulation in *C. roseus* has greatly improved in the past decades. However, there are still quite a few unexplored parts of the pathway. More research is needed for discovering the missing structural genes, enzymes and intermediates of the pathway, as well as genes involved in the regulation of the pathway. This knowledge is needed to develop genetically modified plants, plant cells or microorganisms for the commercial production of the very valuable dimeric alkaloids. So far the genetic modification of the plant, plant cell cultures or microorganisms did not lead to the desired economically feasible production of TIA. In fact it seems that the pathway is more complex than just a series of enzyme catalyzed steps. Compartmentation of the pathway in the plant involves several different cell types, and in these cells of all compartments is one of the complicating factors. This complexity makes it unlikely that a cell suspension culture will be able to perform all steps of the pathway. For a successful biosynthesis all the logistics must be in place, which means that every intermediate and all necessary cofactors and ATP required for the biochemical reactions are present on the right time, in the right quantity, on the right place.

Moreover, the TIA pathway does not exist independently in the total metabolic network of the plant but crosslinks and interacts with other branching pathways, which means it is part of a complex matrix, which raises the question how much of the total carbon flux in the plant can be channeled into TIA biosynthesis. To eventually solve all these problems a systems biology approach is required, which means that all omics will be needed to identify the missing links in the TIA biosynthetic pathway, and map the dynamics of the system. The availability of the full sequences from transcriptomics of many indole alkaloid producing plants under different conditions will be of great use to identify the structural genes of the pathways. The regulatory genes might be more difficult as in the different species the regulation of the biosynthesis can be different. Even in a single plant the regulation will be different between different TIA producing tissues, and between single cells dealing with different parts of the pathway. The single cell approach will thus be a major tool for unraveling

pathways and its regulation and the physiological role of the alkaloids for the plant. Concerning the compartmentation and transport also the single cell analysis will be important as it is likely that the different cell types involved will have different patterns of selective transporters for uptake and extrusion of intermediates in the different cellular compartments and for the whole cell itself. For example, in a leave there will probably be transporters for extrusion of loganic acid in the iridoid producing cells, whereas epidermis cells will have a transporter protein for the selective uptake of this compound. Non alkaloid producing cells may lack this transporter, or have a transporter that extrudes again this intermediate. Also the specific accumulation of the alkaloids in certain cells requires specific uptake and/or extrusion of alkaloids.

There is thus still a long and challenging way to go for complete understanding of the TIA biosynthesis. This knowledge will be of great value for our overall understanding of secondary metabolism in plants. Such research with no doubts will also generate knowledge that can lead to improved production of the dimeric alkaloids, similarly like the finding of the mechanism of the coupling of catharanthine and vindoline (Goodbody *et al.*, 1988) has resulted in an industrial scale chemical production of the dimers from the readily available monomers! This made the monomers as the prime production target instead of the dimeric alkaloids.

Only genetic/molecular tools are not sufficient to figure out the landscape of TIA biosynthesis and regulation. Metabolomics, as a powerful technique to reveal changes in metabolic fluxes, is the ultimate level of post-genomic analysis and facilitates to get a deeper insight in the function of genes and pathways through a systems biology approach. Combination of metabolomics with other "omics" will speed up the elucidation of the TIA pathway and lead to breakthroughs in overcoming the bottlenecks in the production of TIAs in *C. roseus*.

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