# Elucidation of the secoiridoid pathway in Catharanthus roseus

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# Chapter 1

# Monoterpenoid indole alkaloid and iridoid biosynthesis in *Catharanthus roseus*

**Introduction**

Plants produce tens of thousands of different secondary metabolites, compounds with important roles in defence against herbivores, pests and pathogens or interaction with surroundings but not required for plant growth, development or reproduction in controlled conditions. These compounds have a diversity of traditional and novel applications for example as medicines, flavourings, colorants, or in agriculture. *Catharanthus roseus* (Madagascar periwinkle) is one of the most studied medicinal plants and it has been the object of interest of scientists for several decades. It produces the important class of secondary metabolites the monoterpenoid indole alkaloids (MIA) and their precursors the (seco)iridoids. From the over 130 known *C. roseus* MIAs several have pharmaceutical applications such as the anti-hypertensive drugs serpentine and ajmalicine and the potent antitumor agents bisindole alkaloids vincristine and vinblastine that are widely used to treat several types of cancer such as Hodgkins lymphoma, Kaposi's sarcoma, breast cancer, bladder cancer and testicular cancer. Most MIAs are present in plants in very small amounts making their extraction for pharmacological use uneconomical. Because of their complex structures total synthesis is unfeasible (van der Heijden et al., 2004). A lot of effort has been put into biotechnological ways to produce higher amounts of MIAs such as metabolic engineering of cell cultures (Canel et al., 1997), hairy roots (Jaggi et al., 2011; Hughes et al., 2004; Hong et al., 2006; Magnotta et al., 2007; Peebles et al. 2009, 2011; Wang et al., 2010; Pomachova et al., 2009; Zhou et al., 2011; Liu et al., 2011), and even whole plants (Pan et al., 2012) with the help of hormone treatments and intermediate metabolite feeding (Morgan and Shanks, 1999; El-Sayed and Verpoorte, 2002; El-Sayed et al., 2004; Lee-Parsons and Royce, 2006), but a breakthrough still awaits. To date only parts of the biosynthetic pathway leading to MIAs are known. Knowledge of the biosynthesis is essential to biotechnological production of MIAs and (seco)iridoids.

**MIA biosynthesis**

The *C. roseus* MIA biosynthetic pathway is extremely complex with tens of hypothetical steps and different branches. The universal precursor of all MIAs, strictosidine, is produced by condensation of the monoterpenoid secologanin and the indole compound tryptamine (Fig. 1) by the enzyme strictosidine synthase (STR) (Mizukami et al., 1979; Pfitzner and Zenk 1989; McKnight et al 1990; Pasquali et al., 1992; de Waal et al., 1995). Tryptamine originates from the shikimate pathway and is synthesized from tryptophan by the enzyme tryptophan decarboxylase (TDC) (Pennings et al., 1989; De Luca et al., 1989), whereas secologanin originates from the methyl erythritol phosphate (MEP) pathway via the iridoid pathway (Contin et al., 1998; Veau et al., 2000; Chahed et al., 2000; Hong et al., 2003). The iridoid pathway is considered to be the rate-limiting step in MIA biosynthesis (Morgan and Shanks, 1999) and engineering of this part of the MIA biosynthesis pathway may be the key to successfully improve MIA production**.** All MIAs from plants, including the anti-cancer drug camptothecin from *Camptotheca acuminata* and the antimalarial drug quinine from the *Cinchona* tree, are produced from tryptamine and secologanin (O'Connor and Maresh, 2006). This stresses the important role of the MEP and iridoid pathways in engineering of the MIA pathway.

In *C. roseus* MIA biosynthesis occurs via the removal of the glucose moiety from strictosidine by strictosidine-β-D-glucosidase (SGD) yielding strictosidine aglucone giving rise to the highly reactive dialdehyde that can go through a number of molecular rearrangements. This is the first branching point of MIA biosynthesis leading to the tens of *C. roseus* monomeric MIAs (Scott et al., 1977; Luiendijk et al., 1998; Geerlings et al., 2000). Dimeric bisindole alkaloids are formed by condensation of catharanthine and vindoline to form 3',4'-anhydrovinblastine by the peroxidase enzyme α-3',4'-anhydrovinblastine synthase (PRX1) (Costa et al., 2008) further leading to vinblastine and ultimately to vincristine (O'Connor and Maresh, 2006).

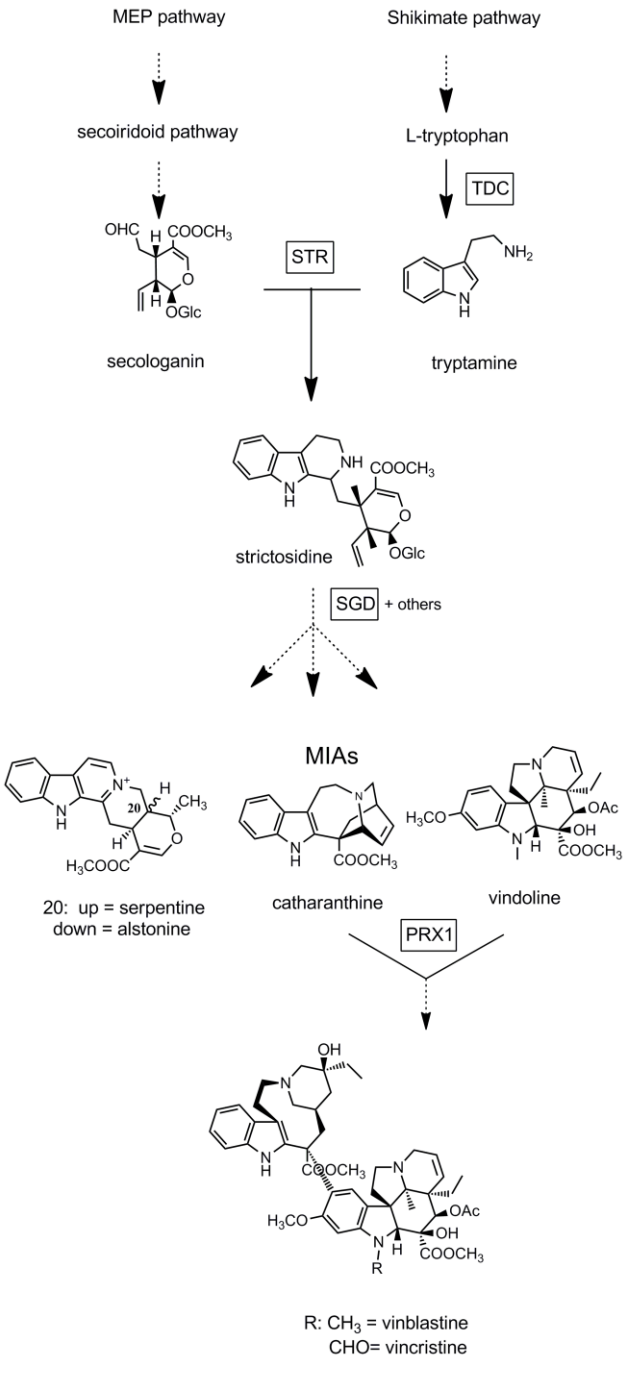


**Fig.1 Overview of MIA biosynthesis**. Solid arrows indicate single enzymatic conversions, dashed arrows multiple reactions. MEP-pathway: methyl erythritol phosphate pathway, TDC: tryptophan decarboxylase, STR: strictosidine synthase, SGD: strictosidine-β-D-glucosidase, PRX1: α-3',4'-anhydrovinblastine synthase.

**Numbering of carbon atoms in terpenoid and iridoid structures**

In literature there exist two different ways of numbering carbon atoms in linear terpenoids. Both are used in this thesis in names of compounds and enzymes (Fig. 1B). One is used mostly in *C. roseus* and MIA biosynthesis related publications (Fig. 2A)(Uesato et al., 1984). The second one used in most of the monoterpene related literature conforms to the naming standards of the international union of pure and applied chemistry (IUPAC), the world authority on chemical nomenclature (IUPAC-IUB, 1986)(Fig. 2B). This nomenclature will be used in the rest of this chapter. As an example 10-OH-geraniol as named acoording to the non-IUPAC nomenclature is 8-OH-geraniol in the IUPAC nomenclature. The cyclic iridoids are usually numbered in a non-IUPAC way (Fig. 2C) (Dinda et al., 2007). Whereas other numbering schemes exist this is the most widely used and is used here for clarity's sake.

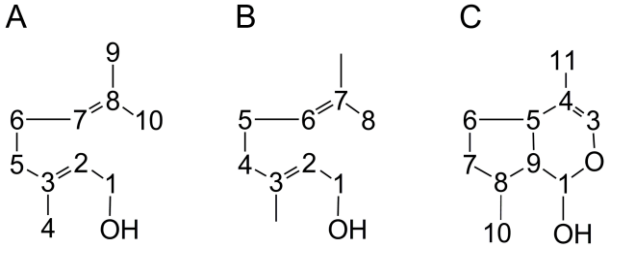


**Fig. 2 Numbering of terpenoid and iridoid carbon atoms**. A: non-IUPAC numbering, B: IUPAC compliant numbering, C: typical numbering of iridoid carbons.

**Monoterpene and iridoid biosynthesis**

Biosynthesis of the terpenoid moiety of MIAs (Fig. 3) starts from the head to tail condensation of dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP)(McGarvey and Croteau, 1995), the basic building blocks of all terpenes, by the enzyme geranyl diphosphate synthase (GPPS) to form geranyl diphosphate (GPP). *C. roseus* has two different kinds of holoenzymes capable of making GPP, the mitochondrial homomeric CrGPPS and the plastidial heteromeric enzyme consisting of a small- and large subunit (CrGPPS-SSU and CrGPPS-LSU). The heteromeric form is thought to supply GPP for MIA production (Rai et al., 2013). The next step is generally assumed to be the synthesis of the acyclic monoterpene alcohol geraniol (Oudin et al., 2007a). While a $Mn^{2+}$ dependent terpene synthase called geraniol synthase (GES) is known to produce geraniol from GPP in *Ocimum basilicum* (Iijima et al., 2004), *Cinnamomum tenuipilum* (Yang et al., 2005), and *Perilla citriodora* (Ito and Honda, 2007) no GES from an iridoid producing plant species such as *C. roseus* had been cloned at the start of the present study.

The first dedicated step in (seco)iridoid biosynthesis is the hydroxylation of geraniol (and its *cis*-isomer nerol) at its 8-position by the cytochrome P450 (CYP) enzyme geraniol-8-oxidase (G8O)(also known as geraniol-8-hydroxylase) (Meehan and Coscia, 1973; Collu et al., 2001) to form 8-hydroxygeraniol (and 8-hydroxynerol respectively). This enzyme was shown to also be able to oxidize the 8-position further to 8-oxogeraniol (Höfer et al., 2013). G8O needs the accessory protein cytochrome P450 reductase (CPR) to function (Madyasha and Coscia, 1979; Meijer et al., 1993). The following steps are less well known, though feeding experiments with (radio)isotopes of intermediates and assays with enzyme preparations from *C. roseus* and other plants suggests oxidation of 8-OH-geraniol/nerol to both 8-oxo-geraniol and 8-hydroxygeranial and further oxidation into 8-oxo-geranial by 8-hydroxygeraniol oxidoreductase (8-HGO) (Uesato et al., 1984, 1986a, 1986b and 1987; Ikeda et al., 1991; Hasnain, 2010). Recently it was found that 8-oxogeranial is turned into the first iridoid, *cis-trans*-nepetalactol (also known as iridodial hemiacetal form), by the iridoid synthase (IS) enzyme in an NADPH dependent reductive cyclization reaction forming the first compound

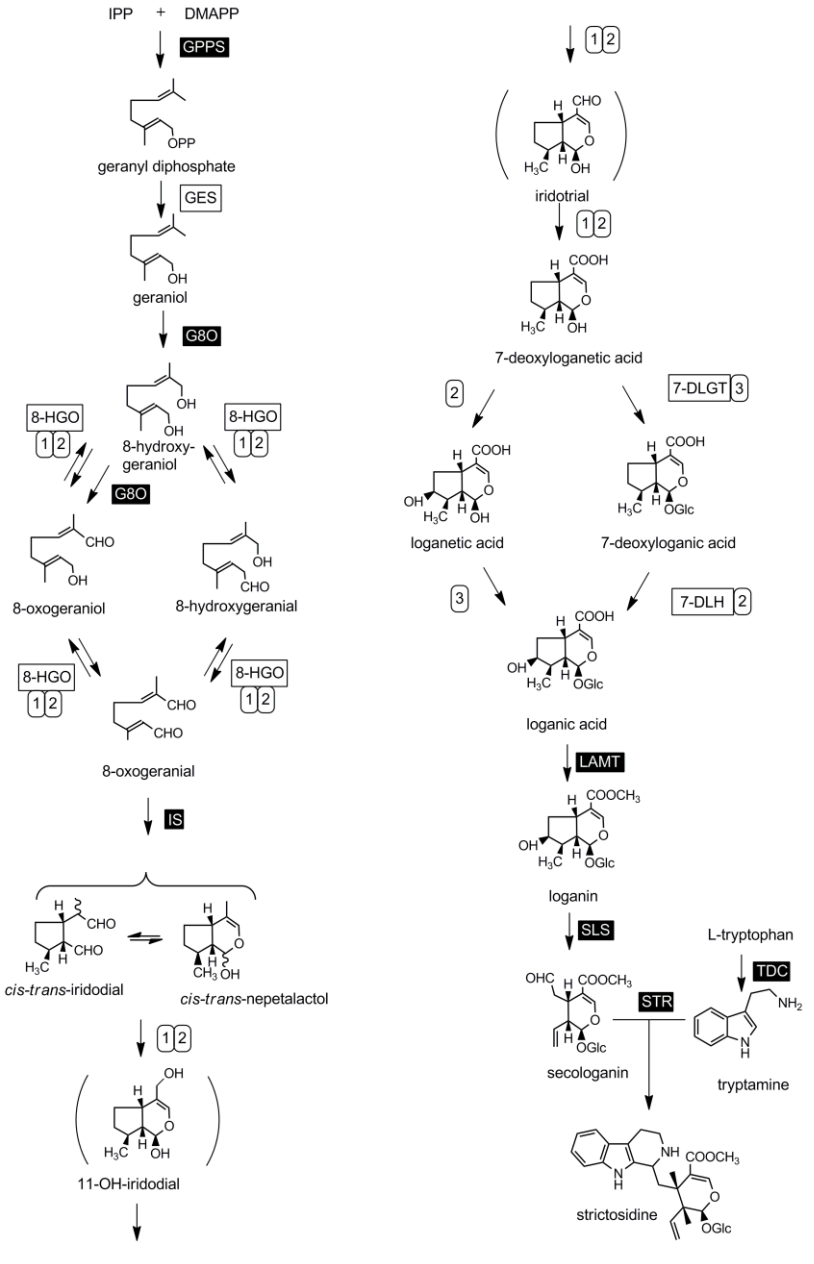


**Fig. 3 Hypothetical model of iridoid biosynthesis.** Solid arrows indicate single enzymatic conversions, dashed arrows multiple reactions. Reactions for which the corresponding *C.roseus* gene has been cloned have a black background, reactions for which the corresponding gene was not cloned but that are described in literature or for which the corresponding gene has been cloned from another organism have a white background. Numbers are for putative enzyme type, 1: oxidoreductase, 2: cytochrome P450, 3: UDP-glucose-glucosyltransferase.

with an iridoid backbone. The compound is considered to exist in equilibrium between the single ring structure *cis-trans*-iridodial and the double ring hemiacetal structure *cis-trans*-nepetalactone (Geu-Flores et al., 2012). Even though a reaction oxidizing cis-trans-nepetalactol at carbon-11 was never described, iridotrial was found to be incorporated into MIAs in isotope feeding experiments making it a plausible intermediate. Genes coding for enzymes catalyzing the later steps have not been cloned but some of the reactions have been characterized from protein extracts. A partially purified enzyme from *Lonicera japonica* cell cultures showed UDP-glucose dependent glucosyltransferase activity by glucosylating 7-deoxyloganetic acid and similar compounds into 7-deoxyloganic and other glucosides, 7-deoxyloganic acid glucosyl transferase (7-DLGT) activity being the most specific (Yamamoto et al., 2002). Nagatoshi et al. (2011) described a UDP-glucose:glucosyltransferase from *Gardenia jasminoides* that glucosylates 7-deoxyloganetin but not 7-deoxyloganetic acid. They also proposed that UGT85A23 (AB591741.1) from *C. roseus* could be the enzyme responsible for iridoid glucosylation. 7-deoxyloganic acid hydroxylase activity (7-DLH), leading to loganin, was observed in an incubation of a microsome preparation from a *Lonicera japonica* cell culture. According to inhibition assays the enzyme is likely to be a CYP (Katano et al., 2001). Loganic acid methyl transferase (LAMT) was cloned from *C. roseus* by Murata et al. (2008). It uses S-adenosyl-methionine (SAM) as co-substrate. Loganin is known to be turned to secologanin by secologanin synthase (SLS), a CYP catalyzing a peculiar ring opening reaction (Yamamoto et al., 1999, 2000; Irmler et al., 2000).

**Tissue and subcellular organization of iridoid biosynthesis**

Many different subcellular compartments in different cell types are required in MIA biosynthesis. This is proposed to minimize the toxic effects of the compounds (Facchini, 2001) but is also seen as a multilayered defence strategy (Guirimand et al., 2010; Roepke et al., 2010). It is speculated that the "Early Steps of Monoterpenoid Biosynthesis" (ESMB), at least until G8O, occur in vascular tissue cells called internal phloem associated parenchyma (IPAP) cells (Burlat et al., 2004; Oudin et al., 2007b). In *C. roseus* the known MEP pathway genes are expressed in IPAP cells and the enzymes located in plastids (Oudin et al., 2007b; Ginis et al., 2012; Burlat et al., 2004; Guirimand et al., 2009). It is not yet known in which cell type the genes coding for the next enzymes, the plastid-located CrGPPS-SSU and CrGPPS-LSU, are expressed but the idea of a co-localized early pathway/ESMB suggests localization in the IPAP cells. Tissue and subcellular localization of GES is also expected to be the same. This would mean that an intermediate metabolite must then be transported to the cytosol since the next enzyme G8O is located on the ER facing to the cytosol, again in the IPAP cells (Burlat et al., 2004; Guirimand et al., 2009). IS reported to be located in the cytosol of IPAP cells (Geu-Flores et al., 2012) suggesting similar localization of the in-between step(s) (8-HGO). Localization of the next enzymes is not known. The later steps in the pathway, LAMT, SLS, TDC and STR, dubbed intermediate pathway, are expressed in epidermal cells (Irmler et al., 2000; Murata et al., 2008; Guirimand et al., 2011b). These enzymes are all localized in the cytosol except STR that localizes to the vacuole. This proposed to have a function in plant defence against herbivores; the glucoside strictosidine is separated from the β-glucosidase SGD as long as subcellular compartments are intact. When cells are disrupted by herbivory, substrate and enzyme mix producing highly reactive and hypothetically toxic protein crosslinking compounds (Guirimand et al., 2010). Dimeric bisindole MIAs are produced in another distinct cell type, i.e. the idioblast/laticifer cells (Guirimand 2011b).

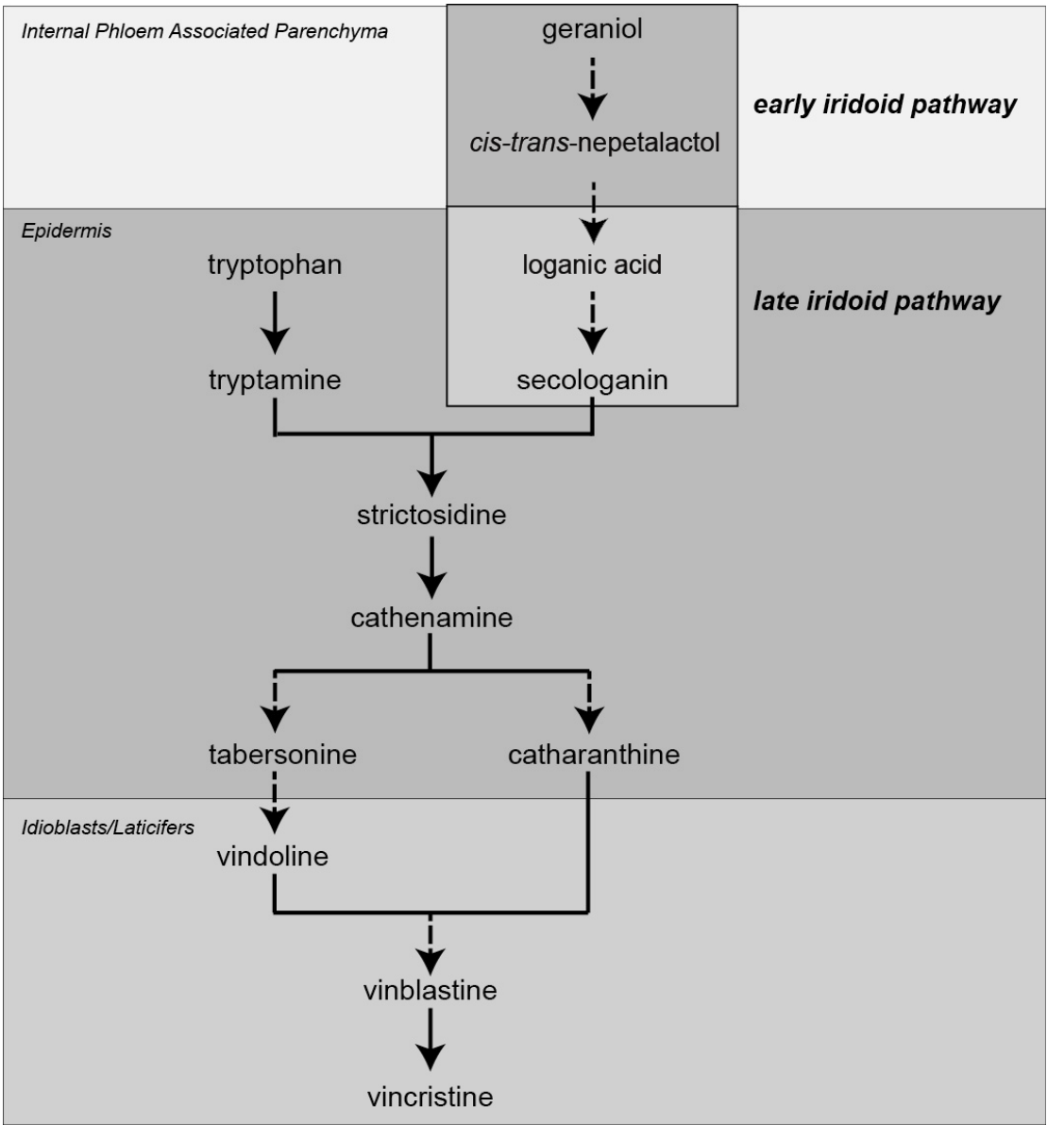


**Fig. 4 Tissue localization of MIA biosynthesis.** Solid arrows indicate single enzymatic conversions, dashed arrows multiple reactions.

**Regulation of MIA and iridoid biosynthesis genes**

MIAs and iridoids are secondary metabolites, compounds that are not regarded as essential for the growth and development of an organism but rather for interaction with its surroundings including as mentioned earlier, plant defence. This is reflected in the way their production is regulated. MIA biosynthetic genes are tightly regulated by developmental cues and external stress signals (Aerts et al., 1994; St. Pierre et al., 1999). A striking mechanism of developmental regulation is that each of the known MIA biosynthetic genes is strictly expressed in one of the three distinct cell types in *C. roseus* aerial organs (IPAP cells, epidermis, idioblasts/laticifers). MIA production is also dependent on the developmental stage of leaves. All known MIA pathway genes are regulated by the defence hormone jasmonate (van der Fits and Memelink 2000; Giddings et al., 2011; Geu-Flores et al., 2012; Oudin et al., 2007; He et al., 2011) orchestrating defence against herbivores, necrotrophic pathogens and biotrophic pathogens. Also the plant hormones auxin, ethylene, cytokinins and the signaling molecule nitric oxide are known to affect the expression of MIA biosynthesis genes and MIA production (Ginis et al., 2012; Yahia et al., 1998; Zhou et al., 2010). Many genes, in the precursor (ASα), intermediate (STR, SLS, LAMT, TDC) and late (D4H) pathway, are regulated by octadecanoid-derivative responsive *Catharanthus* AP2-domain transcription factors (ORCA2, ORCA3) (Menke et al., 1999; van der Fits and Memelink 2000; Hasnain 2010). These jasmonate inducible regulators are known to activate *STR* expression by binding to a GCC box in the jasmonate and elicitor responsive element (JERE) in the STR promoter (Menke et al., 1999; van der Fits and Memelink, 2000; van der Fits et al., 2001). The expression of the ORCA3 gene is controlled by the basic helix-loop-helix (bHLH) transcription factor CrMYC2 that binds an element called a G-box (T/G-box) in the ORCA3 promoter (Zhang et al., 2011). ORCA3 expression is induced by jasmonate via jasmonate dependent degradation of the jasmonate ZIM domain (JAZ) repressors, which bind CrMYC2 in the absence of jasmonate, allowing CrMYC2 to activate the ORCA3 promoter. (Zhang, 2008). The STR promoter also contains a G-box but CrMYC2 cannot activate the promoter directly (Zhang et al., 2011). On the other hand the G-box binding factors CrGBF1 and CrGBF2 act as transcriptional repressors of the STR promoter (Siberil et

al., 2001). A second set of transcription factors, the repressors ZCT1, ZCT2 and ZCT3, bind elsewhere in the STR promoter (Pauw et al., 2004). A similar transcriptional regulation system has been found in tobacco where homologues of ORCA3, the jasmonate inducible NIC2 locus ERF transcription factors activate nicotine biosynthesis gene expression by binding to a GCC box (Shoji et al 2010). ORC1 (ERF221) and ERF189, activate nicotine biosynthesis gene expression but in this case bHLH trancription factors were found to be additionally needed to fully activate expression (Shoji et al., 2011; De Boer et al., 2011). The CrMYC2 homolog NtMYC2 was found to boost activation of nicotine biosynthesis genes by ERF189 by binding a G-box (Shoji et al., 2011) and NbbHLH1 from *Nicotiana benthamiana* was also found to boost activation by ORC1 (De Boer et al., 2011). RNAi silencing of NtMYC2 dramatically reduced the expression of nicotine biosynthesis genes but also the expression of the NIC2 locus ERF genes suggesting that they are regulated by NtMYC2 and leading to a model where nicotine biosynthesis genes are induced by NtMYC2 directly and through ERF transcription factors (Shoji et al., 2011). NtMYC2 itself was found to bind JAZ repressors and be activated by jasmonate via the degradation of the JAZ repressors (Shoji et al 2008). Regulation of *C. roseu*s early pathway genes such as G8O seems to employ a different mechanism and they are known not to be regulated by the ORCAs (Hasnain, 2010). While the G8O promoter does not contain a JERE it also has a G-box sequence (Suttipanta et al., 2007). No transcription factor(s) binding to the G-box in the G8O promoter are known yet but it is likely to be a bHLH, possibly CrMYC2 or a related transcription factor.

**Research strategy and available resources**

This work was part of the SmartCell project, a EU seventh framework funded collaboration between several European groups, where the aim was the elucidation of the secoiridoid pathway and the engineering of the pathway in several organisms for optimized and *de novo* production of iridoids, MIAs and related compounds. A candidate based approach was taken to find enzymes catalyzing both hypothetical and completely unknown intermediate reactions in iridoid biosynthesis and to find suitable enzymes for biotechnical applications. The candidates were picked based on amino acid and nucleotide sequence homology with known enzymes in Leiden and the screening refined by gathering additional information

such as gene expression pattern in different conditions by SmartCell partner Vlaams Instituut voor Biotechnologie, Gent, Belgium, tissue localization of proteins by partners University of Zurich, Switzerland and Université Catholique de Louvain, Belgium and tissue localization of transcripts by the external collaborator Vincent Burlat from University of Toulouse, France. Functional screening of the chosen soluble enzyme candidates was performed in Leiden and P450s were screened by the SmartCell partner Centre national de Recherche Scientifique, Strasbourg, France. Work concerning the *C. roseus* GES was a non-Smart Cell collaboration with Marc Clastre and Andrew Simkin from Tours University, France and collaborators form University of Toulouse, France.

Today's high throughput data mining methods enable the problem to be addressed from multiple angles yielding a broad range of candidates but also the possibility for finer selection and more significant results. Based on the evidence at the onset of these studies the pathway was missing 4-6 enzymes with completely new functionalities. As proposed before (Ikeda et al., 1991; Hasnain, 2010) oxidation of 8-OH geraniol to 8-oxogeraniol should be carried out by a soluble enzyme using $NAD(P)^+$ as a co-substrate by an alcohol dehydrogenase subtype of oxidoreductases expected to be expressed in IPAP cells. Similar reactions have been showed to be catalyzed also by other kinds of oxygenase enzymes such as cytochrome P450s (Höfer et al., 2013), therefore also other types of candidates were taken into account. In order to get the carboxyl group at the 11-position as it is present in the later metabolites, for example in loganic acid, the iridoid intermediate has to be oxidized to form either the hypothetical intermediate 11-OH-iridodial, iridotrial or directly 7-deoxyloganetic acid. While no enzyme types for the reaction have been proposed in literature the hypothetical reaction of 11-hydroxylation is similar to the one of G8O and thus a cytochrome of the CYP76 family seemed a good candidate. In the case of a hydroxylated intermediate an oxidoreductase might be needed to yield iridotrial that can further be oxidized to the carboxylic acid by the same or a different P450. To end up with loganin or any iridoid later in the pathway glucosylation has to happen. This reaction is likely catalyzed by a Plant Secondary Product Glucosyltransferase (PSPG) a subclass of UDP-dependent glucosyltransferases (Vogt et al., 2000; Nagatoshi 2011). Hydroxylation of 7-deoxyloganic acid was previously described as probably done by a P450 (Katano et al., 2001). Candidates

for the *C. roseus* GES were expected to belong to the class of monoterpene synthases identified in several plant species

Several *C. roseus* transcriptomics data sets created with techniques such as cDNA-AFLP (Rischer et al., 2006; Hasnain 2010), random cDNA library sequencing (Murata et al., 2006 and 2008) or next generation sequencing (Van Moerkercke et al., 2013; Gongora-Castilloet al., 2013; Xiao et al., 2013) are currently publicly available. They have been generated from different types of plant material such as different organs/tissues of whole plants, wild type and transgenic cell culture lines and hairy roots in different conditions such as different developmental stages, and with different hormone treatments. This not only gives access to a large amount of sequence data with good coverage of the transcriptome in desired conditions (for example jasmonate treatment) yielding full sequences of candidates, but also enables comparison between datasets and candidate selection based on differential expression. cDNA AFLP data from ORCA2 and ORCA3 inducible overexpression cell culture lines made it possible to screen for ORCA2 and ORCA3 regulated candidates (Hasnain, 2010). Recent proteomics data (Champagne et al., 2012) from MIA producing plant material gives differential data about enzyme distribution over different cell culture lines. As only indirect evidence of the existence of most of the intermediate compounds was available different pathway models were taken into account and the candidate enzymes were assayed with hypothetical intermediates according to the different models. Most compounds to be used in these functional assays were not commercially available so a strategy to obtain such compounds was designed. Some literature on the synthesis exists (Jensen et al., 1987 and 1989; Hasnain 2010) so semisynthesis from natural products according to the literature was chosen as a production method. *Aucuba japonica* leaf material was collected on the Leiden University campus. Aucubine (an iridoid glucoside) was purified by charcoal adsorbtion followed by silica gel chromatography and compound purity was measured with NMR in Leiden. Iridotrial was then produced by Chiralix BV. (Nijmegen, NL) from aucubine by HCOOH,Pd/C reduction and Vilsmeijer formylation. Three other glucosides were produced from iridotrial by organic reduction and oxidation reactions by the same company. Aglucones for enzyme assays were produced by β-glucosidase treatment in Leiden.

In vivo experiments to test candidate genes and known terpenoid and iridoid biosynthesis genes for *in vivo* activity and biotechnological properties in *N. benthamiana, N.*

*tabacum* and *C. roseus*, were performed by SmartCell partners University of Wageningen (WUR), University of Lleida, Spain, VTT Technical Research Centre of Finland and IME Fraunhofer, Aachen Germany. Reconstitution of the pathway in *N. benthamiana* by transient transformation and metabolite analysis of products was conducted at WUR.

**Outline of the thesis**

**Chapter 2** presents cloning and functional characterization of the *C. roseus* geraniol synthase (CrGES). The enzyme expressed in *E. coli* was functionally characterized in *vitro*. Also tissue-specific and subcellular localization of GES were determined. Proof of enzyme in function *in vivo* was given by recombinant expression of CrGES in *Saccharomyces cerevisiae* followed by metabolite analysis. Expression analysis showed it has a jasmonate inducible early monoterpenoid pathway expression pattern.

**Chapter 3** describes cloning of the geraniol synthase from *Valeriana officinalis* (VoGES) and its comparison with the geraniol synthase from *Lippia dulcis* (LdGES). The enzymes expressed in *E. coli* were functionally characterized in *vitro*. GESs were expressed in stable transgenic tobacco lines and transiently expressed in *N. benthamiana* as full length plastidial and N-terminally truncated cytosolic versions and resulting changes in soluble and volatile metabolites were analyzed in detail.

**Chapter 4** presents transcriptomics and proteomics screening of candidates for the missing iridoid pathway enzymes. It describes the characterization of the genes corresponding to all the missing iridoid pathway enzymes, 8-hydroxygeraniol oxidoreductase (8-HGO), 7-deoxyloganetic acid glucosyltransferase (7-DLGT), iridoid oxidase (IO) and 7-deoxyloganic acid hydroxylase (7-DLH) and their functional characterization via *in vitro* enzyme assays. Tissue and subcellular localization is described and proof of function *in vivo* is given by reconstitution of the whole pathway by *Agrobacterium* mediated transient expression *in N. benthamiana.* A detailed metabolite analysis leads to a complete model of the (seco)iridoid biosynthetic pathway.

**Chapter 5** contains a summary and discussion of chapters 2-4

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