The ORCA-ome as a key to understanding alkaloid biosynthesis in Catharanthus roseus

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Chapter 1

Biosynthesis of terpenoid indole alkaloids in *Catharanthus roseus*

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Introduction

Catharanthus roseus is one of the best studied medicinal plants, which produces an important class of plant secondary metabolites known as terpenoid indole alkaloids (TIAs). It is known to biosynthesize more than 130 TIA (van der Heijden et al., 2004). Most of this rich molecular pool has been screened for therapeutics since several decades, and a few of them are marketed (serpentine, ajmalicine and the bisindole alkaloids vinblastine, vincristine and α-3`,4`, anhydrovinblastine). Among these marketed alkaloids vincristine and vinblastine are effective drugs against many types of cancer (van der Heijden et al., 2004). The amount of these compounds in the plant is very low and consequently difficult to isolate (Scott et al., 1979; De Luca and Laflamme, 2001). Plant tissue culture is considered as an alternative source for natural product synthesis because it is independent from environmental factors, climate changes, pests, and geographical and seasonal constraints. Probably due to regulatory constraints alkaloid production is very low or even absent in undifferentiated cells, which clearly is an obstacle for commercial scale production (Verpoorte et al., 2000). Metabolic engineering is thought to be a solution to overcome the rate limiting factors. To overcome the bottlenecks, over-expressing of heterologous or endogenous genes encoding the rate-limiting steps, inactivation of competing pathways, or overexpression of regulatory transcription factors are possible approaches .

The terpenoid indole alkaloid biosynthesis pathway

During the past decades a lot of effort has been put in understanding the biosynthetic pathway of TIA in *C. roseus*. It was found that the biosynthesis of TIA in *C. roseus* is extraordinarily complex and involves the participation of both the shikimate and 1-deoxy-D-xylulose-5-phosphate synthase pathways that yield the required indole and terpene components respectively (Loyola-Vargas et al., 2007). The shikimate pathway consists of a sequence of seven metabolic steps, in which phosphoenolpyruvate and erythrose 4-phosphate are converted to chorismate, the precursor of the aromatic amino acids and many aromatic secondary metabolites. Tryptophan decarboxylase (TDC) catalyzes the conversion of L-tryptophan to L-tryptamine connecting tryptophan to TIA metabolism. Secologanin, a monoterpene glucoiridoid, is condensed with tryptamine to form strictosidine, the universal glucoalkaloid precursor of all terpenoid indole alkaloids found in the plant kingdom. The enzyme that catalyzes the condensation of tryptamine and secologanin is strictosidine synthase (STR).

Terpenoid synthesis

The mevalonate and methyl-erythritol phosphate (MEP) pathways both lead to the terpenoid precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). The mevalonate pathway is active in the cytosol and leads to the formation of triterpenes and sesquiterpenes while the MEP pathway is active in plastids and leads to the formation of monoterpenes, diterpenes and tetraterpenes (Dudareva et al., 2005; Lücker et al., 2007). In *C. roseus* monoterpenoids are produced via the MEP pathway (Fig. 1) (Hong et al., 2003; Oudin et al., 2007). The first step of the MEP pathway is the condensation of pyruvate and glyceraldehyde 3-phosphate by 1-deoxy-D-xylulose-5-

phosphate synthase (DXS) to 1-deoxy-D-xylulose-5-phosphate, DXP. Chahed et al. (2000) isolated and characterised the cDNA of DXS from *C. roseus* The second step is the rearrangement and reduction of DXP by 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), the corresponding cDNA has been cloned from *C. roseus* by Veau et. al. (2000). In the third and fourth step MEP is converted into 4-diphosphocytidyl-2-Cmethyl- D-erythritol (CDP-ME) in a cytidine 5`-triphosphate (CTP)-dependent reaction. The enzyme 4-diphosphocytidyl- 2C-methyl-D-erythritol synthase requires divalent cation (preferably Mg2+) and can use several nucleotide triphosphates besides CTP as substrates. CDP-ME is then phosphorylated on its 2-hydroxy group by a CDP-ME kinase (CMK), which requires magnesium as cofactor

The fifth enzyme (2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase, MECS) converts the 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate into the unusual cyclodiphosphate-containing intermediate 2-C-methyl-D-erythritol 2,4-cyclo diphosphate (Sundberg and Smith, 2002). MECS cDNA was also cloned by Veauet al. (200). An isopentenyl monophosphate kinase catalyzes the last step to IPP and DMAPP. The accumulation of transcripts of DXS, DXR and MECS correlated with TIA biosynthesis in *C. roseus* cell suspension (Hedhili et al., 2007).

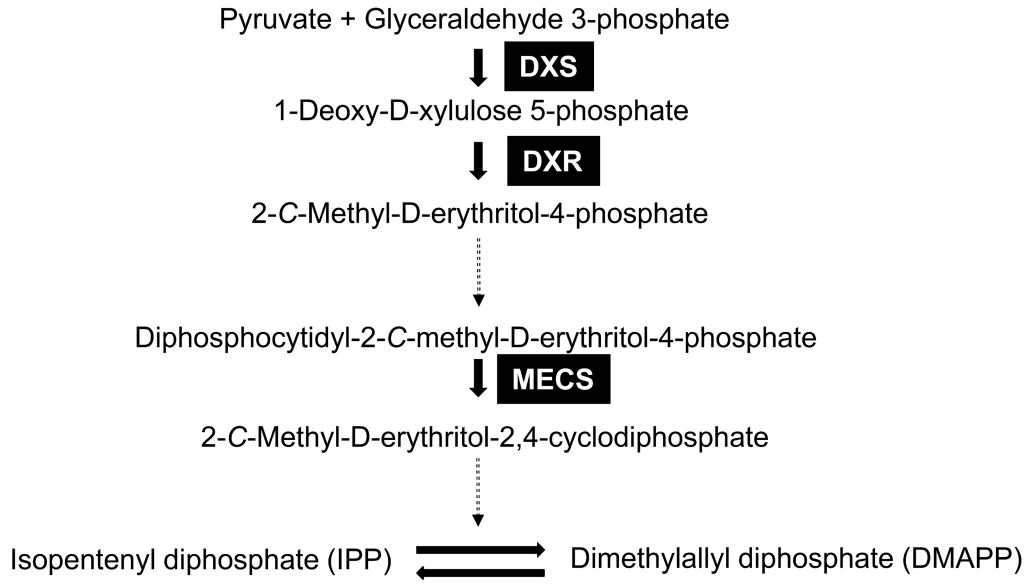

Figure 1. MEP pathway in *Catharanthus roseus.* Solid arrows indicate single enzymatic conversions, whereas dashed arrows indicate multiple enzymatic conversions. Enzymes, for which the corresponding gene was cloned, are indicated. **DXS**: D-1-deoxyxylulose-5-phosphate synthase, **DXR**: 1-deoxy-D-xylulose-5-phosphate reductoisomerase, **MECS**: 2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase

Secologanin synthesis (Terpenoid pathway) Secologanin formation starts from geraniol (Fig. 2). Geraniol is converted to 10-hydroxygeraniol through hydroxylation by enzyme geraniol-10-hydroxylase (G10H), which is a cytochrome P450 enzyme, which needs the help of cytochrome P450 reductase (CPR) to transfer the electron from NADPH (Collu et al., 2001). In the presence of NAD^+ or $NADP^+$, 10-hydroxygeraniol is oxidized into 10-oxogeranial. The enzyme responsible

for this step is a non specific monoterpene-alcohol oxidoreductase (Madyastha and Coscia, 1979). 10-oxogeranial is cyclized by a monoterpene cyclase enzyme, which catalyzes the first cyclization in the biosynthetic pathway of secologanin (Sànchez-Iturbe et al., 2005). The cDNA for this gene is not cloned yet. Iridodial which is the result of cyclization of 10-oxogeranial converts to iridotrial by unknown enzymatic steps. In the formation of 7-deoxyloganine acid from iridodial, so far no enzymes have been described. . Oxidation of the iridotrial aldehyde to the carboxylic acid is followed by esterification and glucosylation to yield deoxyloganin. Subsequent hydroxylation of deoxyloganin yields loganin. Recently the gene loganic acid O-methyltransferase (LAMT) has been cloned and characterized, which catalyzes the reaction to form loganin from loganic acid (Murata et al., 2008). Secologanin is then generated by oxidative cleavage of loganin by the enzyme secologanin synthase (SLS). This NADPH dependent P450 cytochrome was isolated from a cDNA library of an alkaloid-producing *C. roseus* cell culture, and was shown to convert loganin to secologanin *in vitro*, presumably through a radical mediated reaction mechanism (Yamamoto et al., 1999; Irmler et al., 2000). In general, very little is known about the biosynthesis of secologanin. It has been proposed that there are at least 11 enzymatic steps between geraniol and secologanin as final product. However only six are known: geraniol 10-hydroxylase (G10H), $NADP^+$ monoterpene oxidoreductase (10HGO), oxogeranial cyclase, 7-deoxyloganin NADPH:oxygen oxidoreductase (7α-hydroxylase), loganic acid O-methyltransferase (LAMT) and secologanin synthase (SLS).

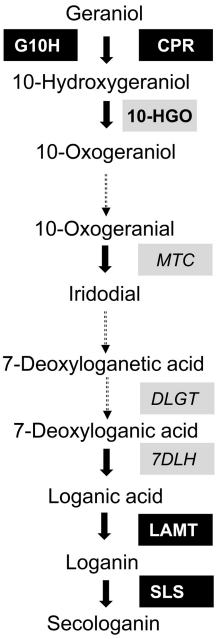

Figure 2. The proposed secologanin biosynthetic pathway in *Catharanthus roseus*. Solid arrows indicate single enzymatic conversions, whereas dashed arrows indicate multiple enzymatic conversions. Enzymes, for which the corresponding gene was cloned are indicated against a black background, and enzymes for which the metabolic step is known in *C. roseus* are shown against a grey background. **G10H**: geraniol-10-hydroxylase, **CPR**: NADPH:cytochrome P-450 reductase, **10-HGO**: 10-hydroxygeraniol oxidoreductase, **MTC**: monoterpene cyclase, **DLGT**: deoxyloganic acid-*O*-glucosyltransferase, **7DLH**: 7-deoxyloganic acid hydroxylase, **LAMT**: loganic acid-*O*-methyltransferase, **SLS**: secologanin synthase

Monoterpenoid alkaloid synthesis

Tryptamine and secologanin are condensed via stereoselective Pictet-Spengler condensation in the first committed step of TIA biosynthesis (Stevens, 1994). In this step, the enzyme strictosidine synthase catalyzes the condensation between tryptamine and secologanin to yield strictosidine (Fig . 3). The enzyme strictosidine synthase (STR) was isolated from cell suspension cultures of *C. roseus* by Mizukami (Mizukami et al., 1979) with an estimated molecular weight between 34 and 38 KDa. Later it was shown that the enzyme STR in *C. roseus* occurs in multiple forms (Pfitzner and Zenk, 1989; de Waal et al., 1995). The correct cDNA sequence and the complete genomic sequence of STR were reported by Pasquali et. al. (1992) and according to their finding this gene occurs as a single copy in *C. roseus*. The different isoforms could be the result of different patterns of glucosylation of the original protein (de Waal et al., 1995) or degradation products during isolation. Strictosidine is converted by the enzyme strictosidine β-D-glucosidase (SGD) to strictosidine-aglycone (Scott et al., 1977; Luijendijk et al., 1998). The cDNA for SGD showed SGD activity when expressed in yeast (Geerlings et al., 2000). Removal of the glucose moiety from strictosidine gives a reactive dialdehyde, which can easily undergo several intra-molecular rearrangements. The first branching point in the TIA pathway is in this stage giving rise to more than a hundred different end products. Since the pathways diverge from strictosidine the enzyme SGD might play a role in determining the kind of alkaloid synthesized (Stevens, 1994). Since the deglucosylation of strictosidine gives rise to a reactive dialdehyde, many compounds are proposed to be formed but *in vitro* cathenamine was found to be the major product. Beside cathenamine, carbinolamine and epi-cathenamine were detected (Stevens, 1994). In other studies, indirect proof was found for the existence of an equilibrium between cathenamine and 4,21-dehydrogeissochizine (Heinstein et al., 1979). Since cathenamine is the main product of strictosidine conversion by SGD, this equilibrium is more favoured towards

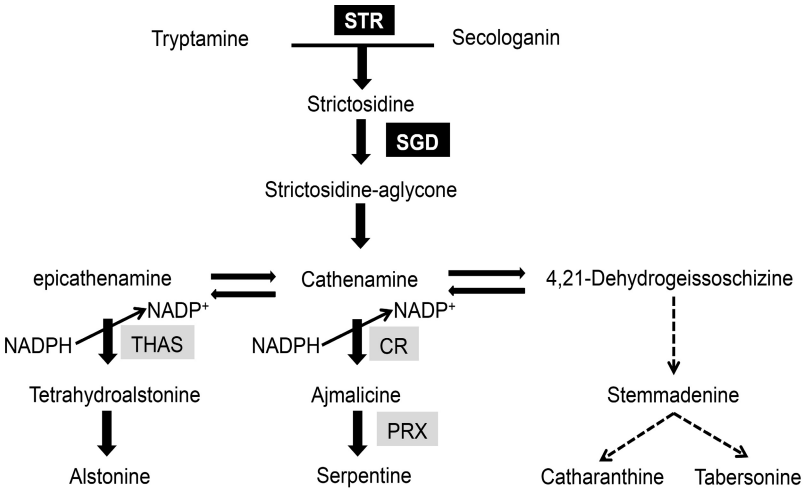

Figure 3. Biosynthetic pathway of terpenoid indole alkaloids in *Catharanthus roseus.* Solid arrows indicate single enzymatic conversions, whereas dashed arrows indicate multiple enzymatic conversions. Enzymes, for which the corresponding gene was cloned are indicated against a black background, and enzymes for which the metabolic step is known in *C. roseus* are shown against a grey background. **STR**: strictosidine synthase, **SGD**: strictosidine β-D-glucosidase, **CR**: cathenamine reductase, **THAS**: tetrahydroalstonine synthase, **PRX**: peroxidase.

cathenamine than to 4,21-dehydrogeissoschizine. Geerlings et al. (1999) found the yellow compound compound dihydroflavopereirine in the reaction mixture after hydrolysis of strictosidine by purified SGD from *C. roseus*.

The studies of the biosynthetic steps directly beyond strictosidine have been restricted to cell suspension cultures of *C. roseus*, which resulted in the conclusion that the *in vivo* conversion of strictosidine results in the formation of 4,21-dehydrogeissoschizine and cathenamine (Stöckigt and Soll, 1980; Zenk, 1980). A partial purified enzyme has been reported that reduced geissoschizine at the C-21α position and thus gives rise to 4,21-dehydrogeissoschizine (Pfitzner and Stoeckigt, 1982). The type of alkaloid formed after strictosidine deglycosylation can be classified on the basis of the rearrangement of the monoterpenoid part into the corynanthe type (e.g. ajmalicine), the aspidosperma type (e.g. tabersonine), or the iboga type (e.g. catharanthine).

The pathway to the corynanthe skeleton from deglycosylated strictosidine starts from the reduction of cathenamine. A partially purified NADPH-dependent reductase isolated from a tetrahydroalstonine-producing *C. roseus* cell line, catalyzed the conversion of cathenamine to tetrahydroalstonine *in vitro* (Hemscheidt and Zenk, 1985). A second *C. roseus* cell line yielded an additional reductase that produced ajmalicine and 19-epi-ajmalicine from cathenamine (Felix et al., 1981). Ajmalicine is further oxidized to yield serpentine by a peroxidise-catalyzed reaction. Although a dedicated enzyme responsible for this oxidation has not been cloned, conversion of ajmalicine to serpentine from peroxidases present in the plant vacuoles has been observed (Blom et al., 1991).

The aspidosperma and iboga type alkaloids are structurally more complex then the corynanthe type and are believed to be derivatives of the corynanthe alkaloids (Qureshi and Scott, 1968). There are reports that the iboga type alkaloid catharanthine as well as the aspidosperma type alkaloid tabersonine are synthesized from the 20,21-aldehyde of dehydrocorynantine and possibly proceeds through stemmadenine (El-Sayed et al., 2004). This finding is based upon feeding of stemmadenine to *C. roseus* cell cultures, which leads to the formation of catharanthine and tabersonine. Nothing is known about the nature of the enzymes involved in the conversion of cathenamine to catharanthine and tabersonine. It was found that addition of methyl jasmonate (MeJA) to cell and root cultures increased the production of catharanthine (Vazquez-Flota et al., 1994). These finding suggest that some of the enzymes participating in the biosynthesis of this alkaloid are subject to regulation which can be used for the study of the catharanthine biosynthesis (Loyola-Vargas et al., 2007).

The biosynthesis of vindoline from tabersonine is composed of six enzymatic steps (Fig. 4). The cytochrome P450 monooxygenase (tabersonine-16-hydroxylase, T16H) is responsible for hydroxylating tabersonine to 16-hydroxytabersonine. The cDNA sequence of this gene was isolated from a cell suspension culture. The recombinant enzyme was able to convert tabersonine to 16-hydroxytabersonine *in vitro* (Schröder et al., 1999). The hydroxyl group is then methylated by a S-adenosyl-L-methionine dependent *O*-methyltransferase to yield 16-methoxy-tabersonine. by the enzyme 16-hydroxytabersonine-16-*O*-methyltransferase (16OMT) (Cacace et al., 2003). The cDNA has been been cloned recently from a cDNA library from leaf epidermal cells (Levac et al., 2008). In the next step, hydration of a double bond by an uncharacterized enzyme produces 16-

methoxy-2,3-dihydro-3-hydroxytabersonine. Transfer of a methyl group to the indole nitrogen by an *N*-methyl transferase (NMT) yields desacetoxyvindoline. This methyl transferase activity has been detected in differentiated plants but not in plant cell cultures (Dethier and Deluca, 1993). The penultimate intermediate, deacetylvindoline, is produced by the action of the 2-oxoglutarate-dependent dioxygenase desacetoxyvindoline 4-hydroxylase (D4H). This enzyme has been cloned (Vazquez-Flota et al., 1997). In the last step, deacetylvindoline is acetylated by deacetylvindoline *O*-acetyltransferase. The cDNA sequence has been isolated from *C. roseus* (St-Pierre et al., 1998).

Dimeric alkaloid biosynthesis

The bisindole alkaloids vincristine and vinblastine are more complex in structure, since they are formed from the dimerization of catharanthine and vindoline. The condensation of catharanthine and vindoline first leads to the formation of the bisindole precursor α-3`,4`-anhydrovinblastine (Scott et al., 1978). This was proved by a feeding experiment in which dehydrovinblastine was incorporated into vinblastine and vincristine by cell free extract from *C. roseus* (Sottomayor and Ros Barcelo, 2003). Peroxidase-containing fractions of plant extracts were found to catalyze the formation of the bisindole dehydrovinblastine from catharanthine and vindoline (Endo et al., 1988), and an enzyme has been purified which catalyzes this step and has been termed α-3`,4`-anhydrovinblastine synthase (PRX1) which belong to class III peroxidase family (Sottomayor et al., 1998). The cDNA sequence has been cloned recently and it was shown that PRX1 can condense catharanthine and vindoline in the presence of H_2O_2 (Costa et al., 2008). Finally, after formation of dehydrovinblastine, hydroxylation of the double bond yields vinblastine, and oxidation of the *N*-methyl group yields vincristine (Sarah and Justin, 2006).

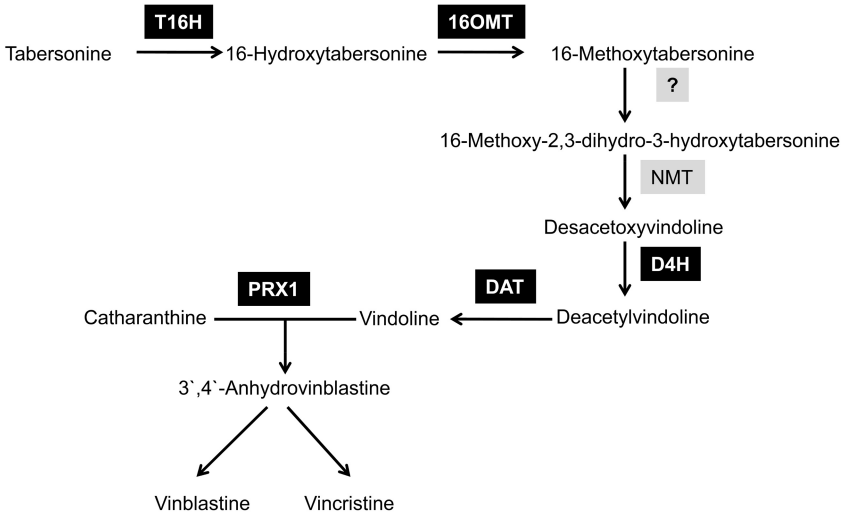

Figure 4. Biosynthesis of vindoline and dimeric indole alkaloids in *Catharanthus roseus*. Solid arrows indicate single enzymatic conversions, whereas dashed arrows indicate multiple enzymatic conversions. Enzymes, for which the corresponding gene was cloned are indicated against a black background, and enzymes for which the metabolic step is known in *C. roseus* are shown against a grey background. **T16H**: tabersonine 16-hydroxylase, **16OMT**:16-hydroxytabersonine-16-*O*-methyltransferase, **NMT**: *N*-methyl transferase, **D4H**: desacetoxyvindoline 4-hydroxylase, **DAT**: acetyl-CoA:4-O-deacetylvindoline 4-O-acetyltransferase, **PRX1**: α-3`,4`-anhydrovinblastine synthase.

Localization of TIA pathway mRNAs and enzymes

The enzymes of the TIA pathway are localized in different parts of the cell and in different tissues of the plant. This compartmentalization is thought to minimize the toxic effect of the compounds to the plant cell (Facchini, 2001) and can be considered as a kind of regulatory mechanism, since this localization requires the transport of different metabolites from one point to another for transformation. Localization studies revealed that TDC, OMT, D4H, and DAT are located in the cytosol. STR and PRX1 are located within the vacuole. On the other hand, G10H, SGD and T16H are located in the endoplasmic reticulum (St-Pierre et al., 1999; Geerlings et al., 2000; Guirimand et al., 2009).

In addition to the compartmentalization of the different enzymatic reactions, another degree of complexity comes from the fact that the enzyme-encoding genes are expressed within different tissues and organs within the plant. The mRNAs for TDC and STR are located in the epidermal cells of stems, leaves and floral buds (St-Pierre et al., 1999). In roots the enzyme TDC is confined to the apoplastic region of the roots, meristematic cells with a slight enrichment in the epidermal cells of the root cap and in the meristematic region (St-Pierre et al., 1999; Moreno-Valenzuela et al., 2003). *DXS*, *DXR*, *MECS*, and *G10H* mRNAs were reported to be expressed in the internal phloem parenchyma and were present in roots, flower buds, and leaves (Burlat et al., 2004). mRNAs for SLS, SGD, T16H and OMT were detected in epidermal cells (Irmler et al., 2000; Murata and De Luca, 2005). G10H expression was also found in epidermal cells (Murata and De Luca, 2005). mRNAs for D4H and DAT were found in the laticifers and idioblasts in the mesophyll of young leaves (St-Pierre et al., 1999).

Regulation of TIA Pathway

Many so-called secondary metabolites are not directly involved in the normal growth and development, but rather are involved in the plant's survival in the natural ecosystem. Terpenoid indole alkaloids are believed to be involved in defence, which is supported by the ways their synthesis is regulated. TIA production is controlled by developmental and exogenous signals. The TIA biosynthesis genes in *C. roseus* are regulated by a number of signals including developmental cues, light, and biotic and abiotic stress. Investigations on seedlings and mature plants revealed that alkaloid synthesis and accumulation is also under developmental control.

The best example of developmentally controlled biosynthesis is the vindoline pathway. Investigation of vindoline biosynthesis through in situ RNA hybridization and immunolocalization experiments revealed that multiple cell types in the leaf and in root tips of *C. roseus* are involved (St-Pierre et al., 1999; Burlat et al., 2004). All known genes related to the biosynthesis of the terpenoid part of TIA biosynthesis, encoding DXS, DXR, MECS and G10H, were shown to be expressed in vascular cells, whereas another set of genes in the later steps of this pathway, encoding TDC, STR and SLS, were expressed exclusively in epidermal cells. On the other hand, two of the genes encoding enzymes involved in the later steps of vindoline biosynthesis, D4H and DAT, were expressed in specialized laticifer and idioblast leaf cells, whereas their expression was never detected in roots. In addition, *TDC*, *STR*, *D4H* and *DAT* genes are expressed at higher levels in the basal part

of the leaf than in the tip, at both mRNA and protein levels, suggesting that their expression is also developmentally regulated (St-Pierre et al., 1999).

Light also has an effect on gene induction. Light exposure of etiolated seedlings activates the expression of the genes *T16H* (St-Pierre et al., 1999), *D4H* (Vazquez-Flota and De Luca, 1998), and *DAT* (St-Pierre et al., 1998).

The production of TIA is also controlled by defense-related signals. The plant hormone jasmonate is a defense signalling molecule in plants and has also a role in development and reproduction. Exogenously added MeJA increased the production of alkaloids by two-fold in *C. roseus* seedlings (Aerts et al., 1994). In *C. roseus* cell lines, the application of MeJA also resulted in an increase in alkaloid production (El-Sayed and Verpoorte, 2002). How jasmonates co-ordinately regulate alkaloid biosynthesis in *C. roseus* has been studied in detail in our lab during the past years.

The promoter of the *STR* gene contains a jasmonate- and elicitor-responsive element (JERE)(Menke et al., 1999). The JERE interacts with two transcription factors called Octadecanoid-Responsive *Catharanthus* AP2-domain (ORCA) proteins. ORCA2 was isolated by yeast one-hybrid screening of a cDNA library with the JERE as bait (Menke et al., 1999) and ORCA3 was isolated by a genetic T-DNA activation tagging approach (van der Fits and Memelink, 2000). Over-expression of ORCA3 in stably transformed *C. roseus* cells led to an increase in the expression levels of *ASα*, *TDC*, *DXS*, *CPR*, *STR*, and *D4H* but not of *G10H, SGD* and *DAT*. On the other hand ORCA2 was found to affect the expression of *STR* and *TDC* but its role in the expression of other TIA pathway genes was not studied in detail.

The expression of the *ORCA2* and *ORCA3* genes is induced by MeJA (Menke et al., 1999; van der Fits and Memelink, 2000). This suggested that the MeJA-responsive increase in expression of alkaloid biosynthesis genes is due to an increase in the amount of ORCA regulatory proteins. However, experiments using the protein synthesis inhibitor cycloheximide showed that *TDC* and *STR* expression does not depend on *de novo* ORCA biosynthesis (van der Fits and Memelink, 2001) and suggest that MeJA activates pre-existing ORCA proteins.

Recently studies of the *G10H* promoter led to the identification of 3 unique regions that could act as potential enhancers. These regions were different from the JERE found in the *STR* promoter. These unique regions point to the possibility of one or more other transcription factors that regulate the activity of *G10H* in response to jasmonic acid, further adding to the complexity of the regulatory network for TIA production (Suttipanta et al., 2007).

In summary in *C. roseus* biosynthesis of TIA is extremely complex. It includes many enzymatic steps, many regulatory check points, and transport of intermediates. Starting from the amino acid tryptophan and the monoterpenoid geraniol, the biosynthesis of bisindole alkaloids in *C. roseus* involves at least 35 intermediates and 30 enzymes (St-Pierre et al., 1999; van der Heijden et al., 2004). Fourteen enzymes for which the corresponding genes were cloned are listed in Table 1, as well as 4 enzymes from primary metabolism involved in precursor production. At least four different subcellular compartments and 3 different tissue types are involved in TIA biosynthesis, and extensive transport of intermediates is required within cells as well as between cells in a plant, based

on enzyme localization and on cell type-specific expression of biosynthesis genes.

Current progress and future prospects of TIA production

Terpenoid indole alkaloids from *C. roseus* are mainly produced and extracted from plants cultivated in fields. Low production of desired TIAs from cultivation prompted the search for alternative approaches, including chemical synthesis and plant cell biotechnology. The chemical structure of bisindole alkaloids is too complex for a complete and economically feasible synthesis. Catharanthine which is produced in plants and cell cultures, and vindoline which is produced in the plant, can be coupled by a chemical or enzymatic reaction to produce vincristine and vinblastine. This technique is used by some companies to produce the drugs. Plant cell biotechnology remains as an alternative source of such metabolites(van der Heijden et al., 2004). A lot of efforts have been made to optimize the culture conditions and ways to increase alkaloid production in *C. roseus* cell lines. Due to the regulatory complexity of alkaloid biosynthesis the yield of alkaloid production in undifferentiated cells is very low or even absent. *C. roseus* cell cultures are a very interesting but so far unsuccessful production system that has been studied for more than three decades (van der Heijden et al., 2004). Metabolic engineering is one of the option which could result in an increased production of TIAs in the whole plant or in cell cultures. Metabolic engineering is defined as the directed improvement of product formation or cellular properties through the modification of specific biochemical reactions or the introduction of a new one, through DNA technology (Stephanopoulos, 1999)

New genes can be introduced in *C. roseus* via *Agrobacterium tumefaciens* or by a DNA-coated particle delivery system. The particle delivery system has been successfully used to generate transgenic *C. roseus* cell lines (van der Fits and Memelink, 1997). Once a gene is introduced into calli, it can be of interest if the cells could be regenerated into transgenic plants, for example if the goal is isolation of alkaloids from field-grown plants as is the current practice. Currently, only Choi et. al. (2004) reported successful regeneration of transgenic *C. roseus* plants from hairy roots. There is no report by others of reproducing the regeneration results. Some key biosynthetic genes have been introduced in *C. roseus* cell lines in attempts to modulate the capacity of TIA production. Overexpression of the *TDC* gene in *C. roseus* crown gall calluses resulted in increased tryptamine content and TDC activity, but TIA contents were not affected (Goddijn et al., 1995). In another case both *TDC* and *STR* genes were introduced in *C. roseus* cells under the control of CaMV 35S promoter through *Agrobacterium*-mediated transformation. The transgenic lines showed a 10 fold increase in STR activity if compared to wild type cells. This resulted in accumulation of elevated levels of strictosidine and other TIAs. On the other hand, high transgene-encoded TDC activity was not only unnecessary for increased productivity, but also detrimental to the normal growth of the cultures (Canel et al., 1998). Anthranilate synthase was introduced in hairy root cultures in an inducible manner. A large increase in tryptophan levels (more than 300-fold) was observed after induction (Hughes et al., 2004). In another study, the cDNA encoding the feedback resistant ASα subunit was introduced alone or in combination with the *TDC* gene via *A. rhizogenes* infection. After induction, the TDC line showed no significant increase in tryptamine levels, whereas the double transgenic line showed a six-fold increase in tryptamine levels. (Hughes et al., 2004; Dong-Hui Liu et al., 2007).

Table 1. Cloned genes encoding enzymes involved in biosynthesis of terpenoid indole alkaloids in *C. roseus*.

Gene	Accession no.	CDS	ORF	Reference
ASα	AJ250008	*1827 bp*	*608 aa*	Bongaerts 1998
DXS	AJ011840	2 151 bp	716 aa	Chahed et al., 2000
DXR	AF250235	1 425 bp	474 aa	Veau et al., 2000
MECS	AF250236	920 bp	236 aa	Veau et al., 2000
CPR	X69791	2 145 bp	714 aa	Meijer et al., 1993
G10H	AJ251269	1 482 bp	493 aa	Collu et al., 2001
10HGO	AY352047	1083bp	360 aa	unpublished
SLS	L10081	1 854 bp	524 aa	Irmler et al., 2000
TDC	X67662	1 503 bp	500 aa	De Luca et al., 1989
STR	X61932	1 280 bp	352 aa	Pasquali et al., 1992
SGD	AF112888	1 857 bp	555 aa	Geerlings et al., 2000
T16H	AJ238612	1 682 bp	495 aa	Schröder et al., 1999
D4H	AF008597	4 232 bp	401 aa	Vazquez et al., 1997
DAT	AF053307	1 320 bp	439 aa	St-Pierre et al., 1998
PRX1	AM236087	1091bp	363 aa	Costa et al., 2008
16OMT	EF444544	1068bp	355 aa	Levac et al., 2008
LAMT	EU057974	1116bp	371 aa	Murata et al., 2008
MAT	AF253415.1	1332 bp	443 aa	Laflamme et al., 2001

The regulatory gene *ORCA3* was introduced into a *C. roseus* cell line under the control of the CaMV 35S promoter by the particle delivery system. Transgenic ORCA3-overexpressing cell lines accumulated significantly more tryptamine and tryptophan, but no changes in TIA content were detected in these cultures. Alkaloid contents were increased when cells were fed with the terpenoid precursor loganin (van der Fits and Memelink, 2000). It was concluded, therefore, that the terpenoid branch of the TIA pathway was limiting TIA production, and that this limitation could not be overcome by *ORCA3* overexpression. A possible bottleneck was *G10H* because *G10H* expression was not observed in the *ORCA3* cell cultures (van der Fits and Memelink, 2000; Memelink et al., 2001).

Successful metabolic engineering is greatly facilitated by the availability of detailed knowledge about the biosynthesis pathway and its regulation. Currently there are large gaps in our knowledge of TIA biosynthesis, as many enzymatic steps are still unknown. The master regulator

ORCA3 regulates the expression of multiple genes in primary metabolism and in the TIA pathway but not all genes. All tested genes in the pathway are responsive to MeJA. This leads to the conclusion that there must be other unknown regulatory proteins which regulate different sets of genes in the pathway. The discovery of new transcription factors, enzymatic steps and their corresponding genes will help us to better understand the TIA pathway and possibly enable engineering of the plant cell in such a way that it can produce the desired alkaloids at elevated levels in an industrial production system.

Outline of thesis

The classic approach to dissect the TIA pathway was based on the identification of a metabolic reaction carried out by a crude protein extract. The identified metabolic reaction can be used to guide the purification of the enzyme. The pure protein can be used to obtain amino acid sequence information or to generate antibodies, which can be used to isolate the corresponding gene. By studying the promoter of that gene the regulatory transcription factors can be identified. In the case of the TIA pathway the regulatory proteins ORCA2 and ORCA3 were identified using the *STR* and *TDC* promoters (Menke et al., 1999; van der Fits and Memelink, 2000). ORCA3 was found to regulate multiple genes in the TIA pathway but the extent to which ORCA2 regulated TIA pathway genes was not studied in detail. The primary objective of this project was to dissect the roles of the transcription factors ORCA2 and ORCA3 in regulation of TIA pathway genes, and to identify new genes in the pathway by using these transcription factors. Overexpression of ORCA transcription factors in a constitutive manner may have deleterious effects on cell growth by producing toxic, or by activation of a negative feedback mechanism. Therefore we used the XVE inducible system (Zuo et al., 2000) to express the ORCAs in *C. roseus* cell lines. The XVE expression system is inducible by estradiol. A schematic diagram of the XVE inducible system is presented in Figure 5.

ORCA2 and ORCA3 were introduced in *C. roseus* cells via particle bombardment. Inducible transgenic GFP lines were also generated as a control. Transcript and metabolite profiling of these transgenic lines and a characterization of a novel TIA biosynthesis gene are presented in the following chapters.

Chapter 2 describes the transcriptomic profiling of transgenic cell cultures overexpressing ORCA2 or ORCA3 in an inducible manner by cDNA-AFLP technology. It was found that ORCA2 and ORCA3 regulate different sets of genes but also an overlapping set of genes.

Chapter 3 presents a study on the effects of a combination of feeding precursors (tryptamine and loganin) and overexpression of ORCA2 or ORCA3 on the TIA biosynthesis, both quantitatively and qualitatively.

Chapter 4 describes that the newly isolated gene CR-75, which is regulated by both ORCA2 and ORCA3, belongs to the NADPH-dependent aldo/keto reductase family of enzymes, and that the encoded protein can convert cathenamine to ajmalicine and hence functions as cathenamine reductase.

In **chapter 5,** the isolation and characterization of a 10-hydroxygeraniol oxidoreductase gene is reported.

Chapter 6 describes the identification and isolation of ORCA2 and ORCA3 regulated esterase enzyme, and an ORCA2 regulated carboxylesterase family of enzymes.

In **chapter 7**, a summary of the results from chapter 2 to 6 is presented.

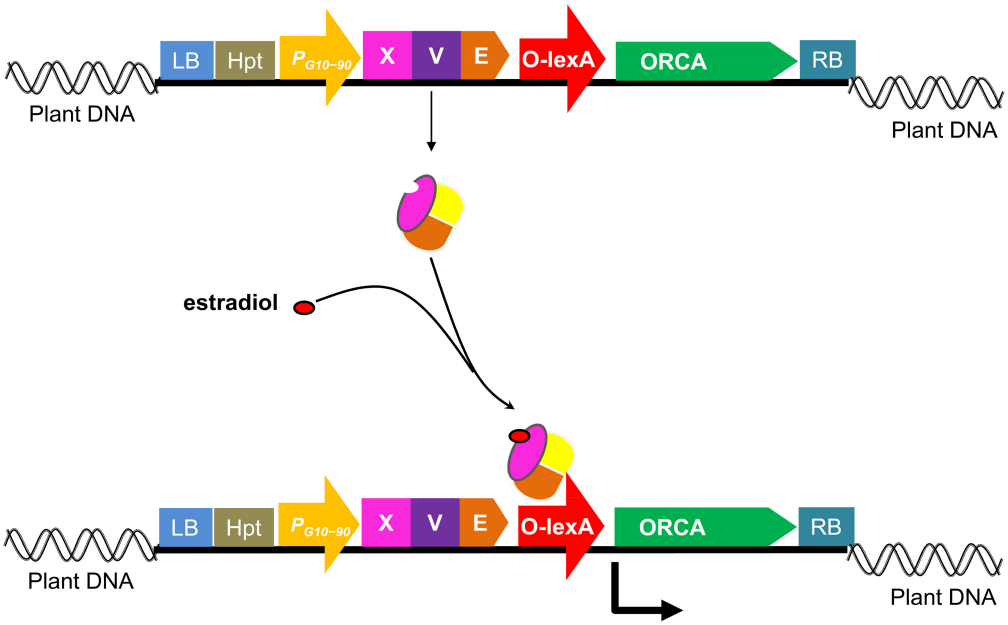

Figure 5. A schematic diagram of the XVE expression system. The region between the right and left borders of the T-DNA is shown (not to scale). P_{G10-90}, a synthetic promoter (Ishige et al., 1999) controlling XVE; XVE, DNA sequences encoding a chimeric transcription factor containing the DNA-binding domain of LexA, the transcription activation domain of VP16 and the regulatory region of the human estrogen receptor; HPT, hygromycin phosphotransferase II coding sequence; O_{LexA}, eight copies of the LexA operator sequence fused to the −46 35S minimal promoter; ORCA transcription factors. Arrows indicate the direction of transcription. Upon binding of estradiol XVE moves to the nucleus.

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