General introduction

Chapter 1

Plant secondary metabolites

Plants are essential to mankind as they are the sources for oxygen, food, clothing, shelter, fuel, and medicine. Unlike human beings, plants are able to produce their own food through photosynthesis, a process of conversion of carbon dioxide and water using energy from the sunlight to make sugars. The sugars are used by plants to synthesize primary metabolites such as starch, pectin, cellulose, fats, lipids, and proteins, which are essential for the cellular processes such as maintenance, growth, and development. Next to these major compounds, there are also minor compounds produced by the plants so-called secondary metabolites that cover a myriad of terpenoids, alkaloids, flavonoids, phenolics, and phenylpropanoids. As the name suggest, secondary metabolites are not of primary importance for the plant growth and development but they play vital roles for the plant survival and their interaction with the environment such as defending against predators and diseases, protecting against abiotic stresses, attracting pollinators and seed dispersal (Dixon 2001; Verpoorte et al. 2002; Verpoorte and Memelink 2002; Sarin 2005; Ramawat 2007). Besides the importance for the plant itself, secondary metabolites are of great benefits to mankind (**Fig. 1**). Many of these plant-derived compounds are used as e.g. medicine, dye, flavor, taste, aroma, fragrance, and insecticide, as a consequence some represent a high economic value (Verpoorte et al. 2000; Verpoorte and Memelink 2002; Verpoorte and ten Hoopen 2006).

The importance of secondary metabolites made these compounds of great interest for mass production. The global market value for plant-derived drugs was $18 billion in 2005 and it is estimated to be more than $26 billion by 2011 (Saklani and Kutty 2008). However, the production of secondary metabolites in the plant itself is often low and their accumulation may be dependent on particular conditions, growth stage, or stress. These compounds are of low molecular weight and some are unique to a plant family or genus or even a species. Many of the valuable medicinal plants producing important drugs can only be grown in certain climates such as tropic or subtropic regions. Some plants grow slowly and some are even difficult to cultivate, thus requiring harvesting from the wild which may result in extinction of the plant species (Alfermann and Petersen 1995; Verpoorte et al. 2002). For these reasons, a biotechnological approach using plant cell or tissue cultures is being explored as alternative

production method of the valuable bioactive metabolites from plants.

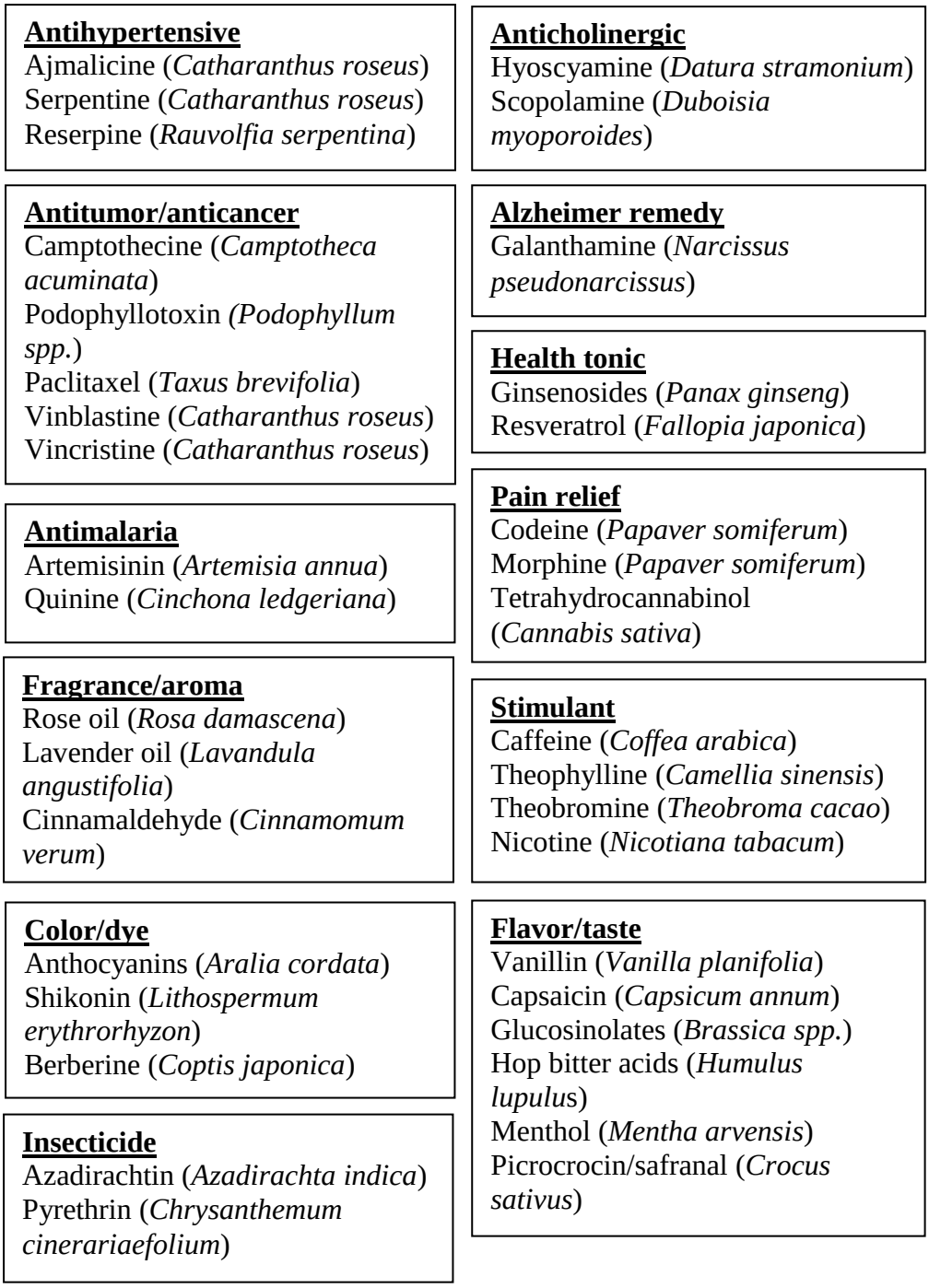

Fig. 1 Examples of plant secondary metabolites used for different purposes and the plant species from which they can be extracted.

Secondary metabolites production by plant cell cultures

As compared to conventional cultivation of intact plants, plant cell cultures have a great potential to be a continuous and reliable source of secondary metabolites. The rapid

multiplication of *in vitro* plant cells is an advantage compared to the slow growth of conventional cultivation of whole plants. Plant cell cultures are cultivated in a controlled environment, thus independent of natural climate changes and are free of microbes and predators. They are thus easy to grow under Good Manufacturing Practice (GMP) for the production of pharmaceuticals. It is easy to maintain the cultures and multiply the cells and subsequently obtain the desired metabolites. Some secondary metabolites produced by the plant cell cultures are shikonin from *Lithospermum erythrorhizon* (Fujita et al. 1981), berberine from *Coptis japonica* (Sato and Yamada 1984), rosmarinic acid from *Coleus blumei* (Gertlowski and Petersen 1993), paclitaxel from *Taxus brevifolia* (Kim et al. 1995), ginsenosides from *Panax ginseng* (Thanh et al. 2005), as well as ajmalicine and serpentine from *Catharanthus roseus* (Zhao and Verpoorte 2007). For commercial production of secondary metabolites, the cell biomass can be scaled-up by cultivating the plant cells in bioreactors. Mitsui Petrochemical Industries (Japan) successfully produced shikonin and berberine in industrial scale using the cell cultures of *L. erythrorhizon* and *C. japonica*, respectively (Fujita 1988). Nitto Denko Corp. in Japan cultivated *P. ginseng* cells in a 20,000 l bioreactor for production of ginsenosides (Alfermann and Petersen 1995; Zhao and Verpoorte 2007). In Korea, root of *P. ginseng* is commercially cultured in 10,000 to 20,000 l specially designed balloon-type bubble bioreactors (Choi et al. 2008). The anticancer drug paclitaxel is produced by Phyton Biotech Inc. (Germany) using *Taxus spp.* cell cultures in 75,000 l bioreactors (Zhao and Verpoorte 2007; Georgiev et al. 2009). Despite a few examples of commercial production by plant cell cultures, still a large number of valuable secondary metabolites are not possible to be produced by plant cell cultures due to the low productivity and high operation costs of the production. One of those extensively studied examples for establishing a production system by plant cell culture is the medicinal plant *Catharanthus roseus*, which represents a rich source of a secondary metabolite group known as terpenoid indole alkaloid.

Catharanthus roseus plant

Catharanthus roseus (formerly named as *Vinca rosea*) originates from the island Madagascar (**Fig. 2**). It is a perennial and evergreen herb which belongs to the family of Apocynaceae. The periwinkle has been widely cultivated and is distributed in all warm and pantropical regions of the world. In Malaysia, *C. roseus* is locally called as Kemunting Cina or Tapak Dara. The subshrub grows about 30 to 100 cm high with glossy and dark green leaves of 2 – 5 cm long and 1 – 3 cm broad. The wild *C. roseus* plant has a pale pink Phlox-

like flower with a purple eye in the center but various cultivars have been developed with flower colors ranging from purple, violet, red, pink, and white. The interest of planting *C. roseus* as an ornamental has increased efforts to breed varieties with more colors, bigger flowers and dwarf plant size. Nowadays, more than 100 cultivars are commercially available (van der Heijden et al. 2004).

Fig. 2 A *Catharanthus roseus* plant.

In addition to its ornamental value, *C. roseus* has long been cultivated as a herbal medicine. It has been used for centuries around the world as remedy for various kinds of ailments such as wasp sting treatment (India); astringent, diuretic, and cough (China); to prevent bleeding (Hawaii); as sore throat remedy and for treating eye infections (Central and South America) and diabetes (Caribbean) (Aslam et al. 2010). There is a report from 1910 stating that *C. roseus* was used in Brazil as a treatment for haemorrhoids and scabies (van der Heijden et al. 2004).

As the leaf extracts of *C. roseus* were well known for its folklore use to treat diabetic, attempts were made to verify the antidiabetic properties of the extracts which led to the discovery of the antineoplastic effect of the plant leaves. The discovery initiated an extensive study towards the anticancer properties of the plant. Subsequently, the activity was found in the alkaloid fraction followed by the isolation of the dimeric terpenoid indole alkaloids vinblastine and vincristine (Noble et al. 1958; Svoboda et al. 1962; Noble 1990). Nowadays, vinblastine (Velbe®) and vincristine (Oncovin®) are commercially available as anticancer

drugs and they are used to treat several types of cancer such as leukemia, Hodgkin's disease, Wilm's tumors, testicular and breast cancers. Semi-synthetic derivatives of vinblastine and vincristine have been developed as potent anticancer agents (van der Heijden et al. 2004).

Next to the anticancer compounds, *C. roseus* also produces two antihypertensive alkaloids ajmalicine and serpentine, which are applied as pharmaceuticals. In addition, the plant produces more than 130 terpenoid indole alkaloids (TIA), thus making *C. roseus* a rich source of TIA (Moreno et al. 1995; van der Heijden et al. 2004). However, most TIA are present in very small amounts, especially the dimeric/bisindole alkaloids. Thus large quantities of raw material are needed for compound isolation. For example, to isolate 1 g of vinblastine, about 500 kg of *C. roseus* leaves are required (van der Heijden et al. 2004). This low yield and consequently the high market price are a major constraint for the clinical use of this important drug. Furthermore, plant compounds like ajmalicine and vinblastine have complex structures, thus commercial total synthesis of these compounds is not feasible and the supply of the drugs is based on the cultivated plant. Although it is possible to semi-synthesize vinblastine from its monomeric precursors catharanthine and vindoline, the supply of these precursors is also from the plant and needs to be further improved. To produce higher amount of TIA and precursors from *C. roseus*, considerable efforts have been put into developing a biotechnological production.

Catharanthus roseus cell cultures: a biotechnological approach

Plant biotechnology promised interesting opportunities for the production of phytochemicals. But due to its low productivity of important alkaloids, much work has been done on the optimization of the cell culture systems and *C. roseus* became a model plant for biotechnological studies. It is now one of the best-studied medicinal plants in plant biotechnology research. The goal of the biotechnological studies is to increase the yield of TIA by large-scale culture of *C. roseus* cells. Technically, the large-scale cultivation of *C. roseus* cell culture is possible, however TIA production in the cell cultures is too low or for some even completely zero. The process operation costs are too high for commercialization (Verpoorte et al. 1999; 2002). Several aspects of biotechnological production of *Catharanthus* alkaloids have been discussed by van der Heijden et al. (1989), Moreno et al. (1995), van der Heijden et al. (2004), Verpoorte et al. (2002), Zhao and Verpoorte (2007), Zhou et al. (2009), and Mujib et al. (2012). Different strategies have been applied to increase the production of TIA in *C. roseus* cells, such as 1) screening and selection for high TIA-

producing cell lines; 2) optimization of culture conditions; 3) differentiated cell and organ cultures; 4) feeding and elicitation techniques; and 5) metabolic engineering.

Screening and selection of the cell lines are classical approaches for optimization of biotechnological production systems. Different *C. roseus* cell lines have different productivity ranging from low-, to high-, or even non-TIA producing cell lines. Therefore, selection of cell lines with suitable biochemical and physiological characteristics is an important approach to improve the productivity for the targeted compounds (Zhao and Verpoorte 2007). However, plant cell cultures are often genetically unstable during long term maintenance and this may affect the production of secondary metabolites which requires resources not available for growth. In addition, screening for the high-producing cell lines is quite laborious and time consuming, but an increased productivity of 10 – 20 fold is feasible by this method (Verpoorte et al. 1998).

Optimization of the culture medium, plant growth regulators, and culture conditions were extensively studied to improve the cell biomass accumulation and the TIA production (reviews in van der Heijden et al. 1989; Moreno et al. 1995; Zhao and Verpoorte 2007; Zhou et al. 2009; Mujib et al. 2012). An improvement of TIA productivity by 20 – 30 fold could be obtained by combination of such optimizations and selection of high-yielding cell lines. However, this approach is limited to the cell cultures which have the targeted compounds and it does not work if the compounds of interest are not present at all in the cell cultures like in case of the bisindole alkaloids vinblastine and vincristine (Verpoorte et al. 1999; 2002).

Secondary metabolites are often produced in specific tissues and the biosynthesis involves cellular compartmentation. The highly valuable anticancer compounds vinblastine and vincristine did not accumulate in *C. roseus* cell cultures due to the lack of vindoline, one of the precursors of the bisindole alkaloids. Vindoline is accumulated in the plant leaves and requires functional chloroplasts for one of the steps in its biosynthesis (De Luca and Cutler 1987; De Luca et al. 1987). Its biosynthesis also involves organization of particular cell types found in the aerial tissues (St-Pierre et al. 1999; Verma et al. 2012; Salim and De Luca 2013). Therefore, differentiated cells or organ cultures, such as shoot cultures could be an alternative for producing vindoline and dimeric/bisindole alkaloids which are absent in undifferentiated cell cultures. Similarly, compounds found in the roots can be produced by root cultures. However, such organ cultures are difficult to grow on a large-scale and require special designed bioreactors, such as a rolling drum bioreactor, mist bioreactor, airlift bioreactor, bubble column bioreactor, balloon-type bubble bioreactor, temporary immersion bioreactor,

or plastic disposable bioreactor (Verpoorte et al. 1999; Choi et al. 2008; Ducos et al. 2008; Eibl and Eibl 2008).

Though vindoline is not accumulated in the *C. roseus* cell cultures, catharanthine could be synthesized at much higher level in the cell cultures compared to the plants (Misawa and Goodbody 1996). As the bisindole alkaloids are only minor products in the plant, these compounds can be produced in a semi-synthetic way by coupling catharanthine and vindoline produced by the cell or organ cultures. Therefore, the biotechnological approach using separate cell or organ cultures could be promising sources of catharanthine and vindoline, the precursors of the important drugs vinblastine and vincristine. However, there is still a challenge to make these precursors as major products in the cell or organ cultures due to the low productivity. Extensive research is needed to learn more on the regulation of the biosynthetic pathway to apply this knowledge to novel approaches to increase the flux towards the compounds of interest (Zhao and Verpoorte et al. 2007).

In order to evaluate the flux limiting steps of the TIA pathway, precursor feeding studies were performed in the *C. roseus* cell cultures. TIA derives from precursors of two biosynthetic pathways, the terpenoid-iridoid pathway and the shikimate-tryptophan pathway. Some studies found that feeding tryptophan or tryptamine in *C. roseus* cell cultures increased, decreased, or had no effect on TIA levels (e.g. Döller et al. 1976; Krueger and Carew 1978; Merillon et al. 1986; Zenk et al. 1977; Knobloch and Berlin 1980; Contin et al. 1999; Whitmer et al. 2002a). On the other hand, feeding the iridoid precursors loganic acid, loganin, and secologanin to *C. roseus* cell cultures increased the TIA level. Among the iridoid precursors feeding, loganin fed-cells gave the highest TIA accumulation. Also feeding studies with elicited cells or transgenic cell lines showed that TIA production is limited by availability of the precursor from the terpenoid pathway (Moreno et al. 1993; Whitmer et al. 1998, 2002a, 2002b).

Several studies were also conducted on the effect of feeding upstream precursors of the terpenoid pathway, such as geraniol and mevalonic acid. Feeding geraniol in *C. roseus* cell suspension cultures did not show an effect on TIA production (Krueger and Carew 1978), but another study showed an increase of ajmalicine production (Lee-Parsons and Royce 2006). In addition, feeding geraniol to *C. roseus* hairy roots increased the tabersonine level (Morgan and Shanks 2000). Although a ^{14}C-label from mevalonate was incorporated into the iridoids moiety (Guarnaccia et al. 1974), studies showed that feeding mevalonic acid had no effect on TIA levels (Krueger and Carew 1978; Moreno et al. 1993; Morgan and

Shanks 2000), which is explained by the fact that the iridoid pathway derives from the MEP-terpenoid pathway (Contin et al. 1998).

Elicitation is one of the strategies to stimulate product formation in plant cell cultures. Various abiotic and biotic elicitors can be used to induce the biosynthesis of secondary metabolites (van der Heijden et al. 1989; Moreno et al. 1995; Namdeo 2007; Zhao and Verpoorte 2007). The signal molecule jasmonic acid or its methyl ester are commonly applied to increase TIA accumulation in *C. roseus* cell cultures (El-Sayed and Verpoorte 2002; Lee-Parsons and Royce 2006; Vázquez-Flota et al. 2009). The increased levels of TIA upon jasmonate elicitation result from elevated expression of a set of TIA biosynthesis related genes which is controlled by transcriptional regulators known as octadecanoid-derivative responsive *Catharanthus* AP2-domain (ORCA) proteins (Memelink et al. 2001). The significant induction of the TIA pathway after jasmonate elicitation suggests the involvement of TIA in the plant defense responses, as supported by reports which showed that TIA have antimicrobial and antiherbivore activity (Luijendijk et al. 1996; Guirimand et al. 2010). The application of jasmonates to *C. roseus* cell cultures not only improves TIA production, but is also useful in studying signal transduction and regulatory mechanisms underlying the induction of TIA biosynthesis (Zhao and Verpoorte 2007). Genome-wide transcript profiling by cDNA-amplified fragment-length polymorphism combined with metabolic profiling of jasmonate elicited *C. roseus* cell cultures yielded a collection of known and previously undescribed transcript tags and metabolites associated with TIA (Rischer et al. 2006).

Since the cell cultures of *C. roseus* are unable to reach a sufficiently high level for commercial production, research has been focused more on the regulation of the biosynthetic pathways and strategies to engineer the plant cell factory itself rather than engineering the biochemical process (Zhao and Verpoorte 2007). Engineering the metabolic fluxes by manipulating and controlling the metabolic pathways towards targeted compounds seems promising to achieve a commercially viable TIA production. To achieve this goal, one or a combination of the following approaches can be applied: 1) overcome rate-limiting steps by overexpressing one or multiple genes of the biosynthetic pathway or overexpressing transcription factors that control multiple pathway genes; 2) suppress competitive pathways that share the same precursor pool with the TIA; 3) suppress catabolism of the product of interest. Competitive pathways and catabolism can be suppressed by antisense genes, RNAi methods, or using antibodies. More often, metabolic engineering of *C. roseus* cell cultures, hairy roots, or plants is performed by overexpressing biosynthetic genes or regulatory genes

of the TIA pathway. Some genes involved in TIA biosynthesis have also been transferred to heterologous hosts such as tobacco and yeast (review in Verpoorte et al. 1999; Verpoorte and Memelink 2002; van der Heijden et al. 2004; Zhao and Verpoorte et al. 2007; Salim and De Luca 2013; Pan 2014). Yet, this attempt requires thorough knowledge about biosynthetic routes and intermediates, as well as the enzymes and their encoding genes. Regulatory aspects, compartmentation, and transports are the other factors that should also be considered for engineering the TIA metabolism. In fact it is now obvious that simple metabolic engineering just involving a few steps will not lead to the desired increase of alkaloid production. A synthetic biology approach engineering the whole network involved in TIA biosynthesis, including engineering cell physiology is the next step. Therefore, further insight in the total regulation of TIA biosynthesis and related pathways is needed.

Biosynthesis of *Catharanthus* alkaloids

A complete knowledge of the biosynthetic pathway of the targeted compounds and its regulation is essential to increase the metabolic flux towards the desired products. The biosynthesis of *Catharanthus*' alkaloids has been studied extensively though not all parts of the biosynthesis are yet elucidated due to its complexity. The alkaloids in *Catharanthus* belong to the group of terpenoid indole alkaloid (TIA) as their biosynthetic building blocks are a monoterpenoid (secologanin) and an indole (tryptamine) (**Fig. 3**). Tryptamine is synthesized from tryptophan (product of the plastidial shikimate pathway) by the enzyme tryptophan decarboxylase (TDC) (Pennings et al. 1989; De Luca et al. 1989), whereas secologanin originates from the monoterpene geranyl diphosphate (GPP) in the plastidial methyl erythritol phosphate (MEP) pathway (Contin et al. 1998; Hong et al. 2003). These two compounds are the universal precursors of all TIAs in plants including the antihypertensive reserpine from *Rauvolfia serpentina*, the antineoplastic camptothecin from *Camptotheca acuminata*, and the antimalarial alkaloid quinine from *Cinchona ledgeriana* (O'Connor and Maresh 2006).

TIA biosynthesis starts with the condensation of the monoterpenoid secologanin and the indole compound tryptamine by the enzyme strictosidine synthase (STR) to produce strictosidine, the central intermediate of all TIAs (Mizukami al. 1979; Pfitzner and Zenk 1989; De Waal et al. 1995). Subsequently, deglucosylation of strictosidine by the enzyme strictosidine-*β*-D-glucosidase (SGD) forms a reactive strictosidine aglucon, which is converted into cathenamine (Luijendijk et al. 1998; Geerlings et al. 2000; Guirimand et al. 2010). Cathenamine is quite a reactive carbinolamine and can be further converted into

different basic TIA skeletons (Stöckigt et al. 1977). Reduction of cathenamine by the enzyme cathenamine reductase (CR) produces ajmalicine, which upon further oxidation by class III peroxidases leads to the formation of serpentine (Blom et al. 1991; Sottomayor et al. 2004). In contrast, the iminium form of cathenamine, epicathenamine, produces tetrahydroalstonine by tetrahydroalstonine synthase (THAS) and which can be further oxidized into alstonine. The reversible intermediate 4,21-dehydrogeissoschizine can also be converted into stemmadenine which leads to the production of vindoline and catharanthine, the monomeric precursors of bisindole alkaloids vinblastine and vincristine (El-Sayed and Verpoorte 2007).

At present, no genes, enzymes, or intermediates involved in the catharanthine pathway have been characterized. On the other hand, the biosynthesis of vindoline is quite well characterized. It involves six sequential enzymatic reactions starting from the intermediate tabersonine, in which five of the enzymes have been purified. The first reaction is hydroxylation of tabersonine to 16-hydroxytabersonine by tabersonine 16-hydroxylase (T16H). Subsequently, the enzyme 16-hydroxytabersonine 16-*O*-methyltransferase (OMT) catalyzes the methylation towards 16-methoxytabersonine. The hydration of 16-methoxytabersonine to 16-methoxy-2,3-dihydro-3-hydroxytabersonine remains yet uncharacterized, whereas further steps to desacetoxyvindoline and deacetylvindoline are catalyzed by *N*-methyltransferase (NMT) and desacetoxyvindoline 4-hydroxylase (D4H), respectively. Deacetylvindoline 4-*O*-acetyltransferase (DAT) catalyzes the last step of the pathway to form vindoline (De Carolis et al. 1990; Power et al. 1990; Dethier and De Luca 1993; De Carolis and De Luca 1993; St-Pierre and De Luca 1995; El-Sayed and Verpoorte 2007). The first two enzymes, T16H and 16OMT are present in the plant cell cultures, whereas distribution of NMT, D4H, and DAT is restricted in particular cells (laticifer and idioblast) in the leaves (St-Pierre and De Luca 1995; Schröder et al. 1999; Verma et al. 2012). In contrast to vindoline formation via tabersonine in aerial tissues, lochnericine and hörhammericine are accumulated in roots via tabersonine metabolism. Hörhammericine is converted into 19-*O*-acetylhörhammericine by the enzyme miniovincinine-19-hydroxy-*O*-acetyltransferase (MAT). Alternatively, 19-*O*-acetylhörhammericine is produced from tabersonine via 6,7-dehydrominiovincinine (Verma et al. 2012; Salim and De Luca 2013).

Oxidative coupling of the monomeric vindoline and catharanthine produces the bisindole alkaloid α-3',4'-anhydrovinblastine. The dimerization step is catalyzed by α-3',4'-anhydrovinblastine synthase known as CrPrx1, which belongs to the class III basic peroxidases (Sottomayor et al. 1998; Costa et al. 2008). The last steps towards vinblastine and vincristine are still uncharacterized. However, it was proposed that an iminium ion can be

formed as an unstable intermediate during the dimerization step, and both anhydrovinblastine and the iminium ion can be incorporated into vinblastine and vincristine (Sottomayor et al. 2004; Salim and De Luca 2013).

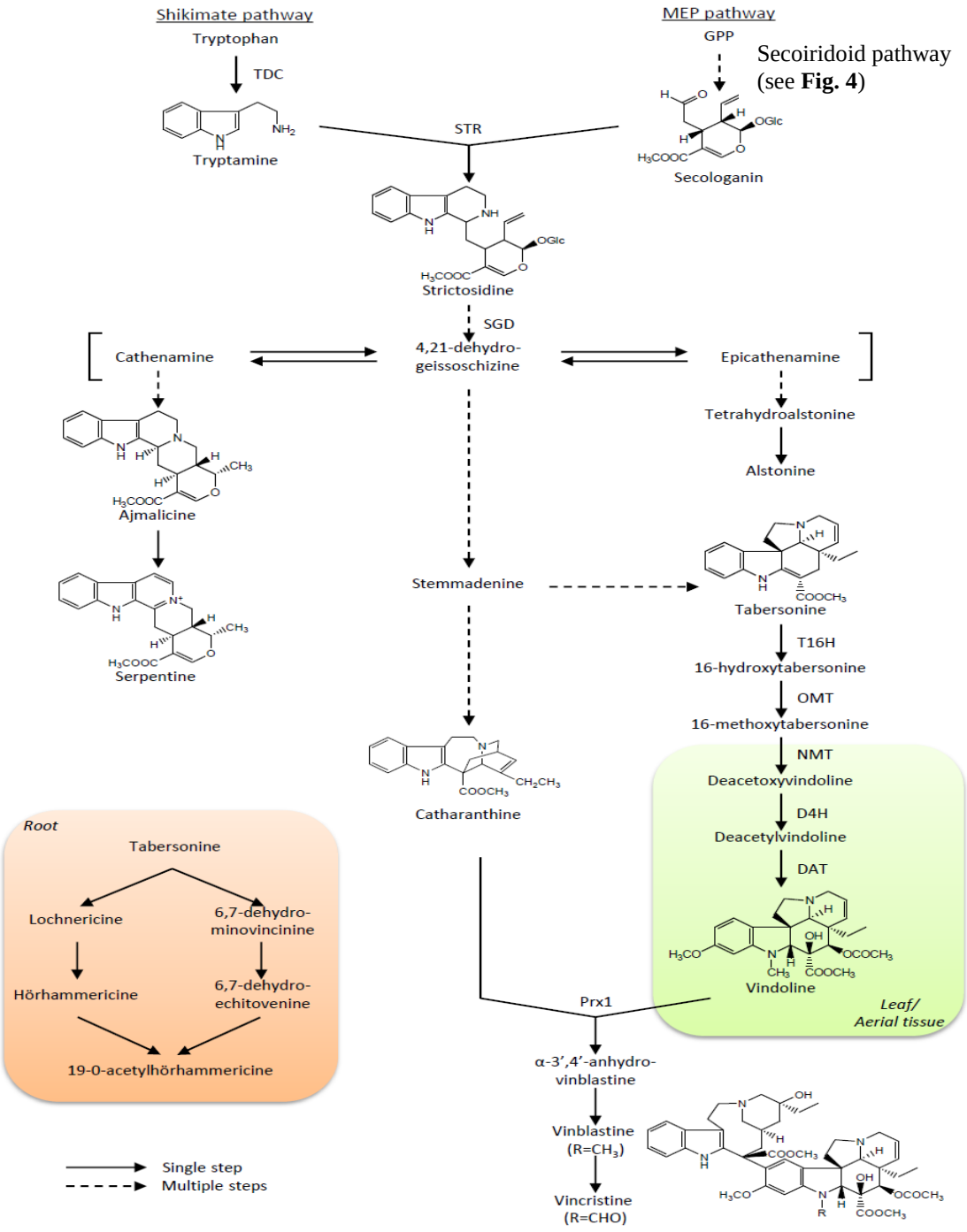

Fig. 3 Biosynthetic pathway of terpenoid indole alkaloids in *Catharanthus roseus* leading to the anticancer compounds vinblastine and vincristine. D4H: desacetoxyvindoline 4-hydroxylase, DAT: deacetylvindoline 4-*O*-acetyltransferase, GPP: geranyl diphosphate, NMT: *N*-methyltransferase, OMT: 16-hydroxytabersonine 16-*O*-methyltransferase, PRX1: peroxidase 1, SGD: strictosidine *β*-D-glucosidase, STR: strictosidine synthase, T16H: tabersonine 16-hydroxylase, TDC: tryptophan decarboxylase.

Monoterpenoid-iridoid pathway in *Catharanthus roseus*

The central intermediate of all TIAs, strictosidine, is a product of condensation between the iridoid secologanin derived from the monoterpenoids in the MEP pathway and the indole alkaloid tryptamine from the shikimate pathway. The monoterpenoid/iridoid pathway is considered the rate-limiting step of TIA production in *C. roseus* cell cultures (Moreno et al. 1993; Whitmer et al. 2002b). Therefore, the engineering of the metabolic flux in this pathway seems an interesting approach to improve the production of TIA. However, the lack of knowledge of the iridoid biosynthesis was the main obstacle to engineer the pathway. Recently, the missing intermediates and enzymes in the secoiridoid pathway have been identified by Miettinen et al. (2014) (**Fig. 4**). This opens new possibilities to overcome the rate-limiting steps and possibly increase TIA production.

The terpenoid moiety of TIA derives from geranyl diphosphate (GPP; C10), the precursor for all monoterpenoids. GPP is produced from the C5 precursors isopentenyl diphospate (IPP) and dimethylallyl diphosphate (DMAPP) by the enzyme geranyl diphosphate synthase (GPPS) (Rohmer et al. 1999). There are two different types of GPPS in *C. roseus*, i.e. 1) mitochondrial homomeric CrGPPS and 2) plastidial heteromeric CrGPPS.LSU (long subunit) and CrGPPS.SSU (short subunit), in which the heteromeric GPPSs provide GPP for TIA formation (Rai et al. 2013). GPP is converted into geraniol by the enzyme geraniol synthase (Simkin et al. 2013). Subsequently geraniol is hydroxylated to form 8-hydroxygeraniol (also known as 10-hydroxygeraniol) by the cytochrome P450 enzyme geraniol 8-oxidase (G8O), also known as geraniol 10-hydroxylase (G10H) (Collu et al. 2001). The G8O requires a cytochrome P450 reductase (CPR) to function (Madyastha and Coscia 1979; Meijer et al. 1993). The enzyme 8-hydroxygeraniol oxidoreductase (8-HGO) catalyzes the oxidation of 8-hydroxygeraniol at both C1 and C8 to form 8-oxogeranial (Miettinen et al. 2014). Cyclization of 8-oxogeranial into iridodial is catalyzed by iridoid synthase (IS), a cyclase recruited from short-chain oxidoreductase (Geu-Flores et al. 2012). A cytochrome P450 iridoid oxidase (IO) turns cis-trans-iridodials and cis-trans-nepetalactol into 7-deoxyloganetic acid, which subsequently forms 7-deoxyloganic acid by the enzyme 7-deoxyloganetic acid glucosyltransferase (7DLGT). The latter compound is hydroxylated by 7-deoxyloganic acid hydroxylase (7DLH) into loganic acid (Miettinen et al. 2014). Loganic acid is methylated by loganic acid methyltransfrase (LAMT) into loganin (Murata et al. 2008) and secologanin synthase catalyzes the conversion of loganin into secologanin, the terpenoid precursor for TIAs (Yamamoto et al. 1999, 2000; Irmler et al. 2000).

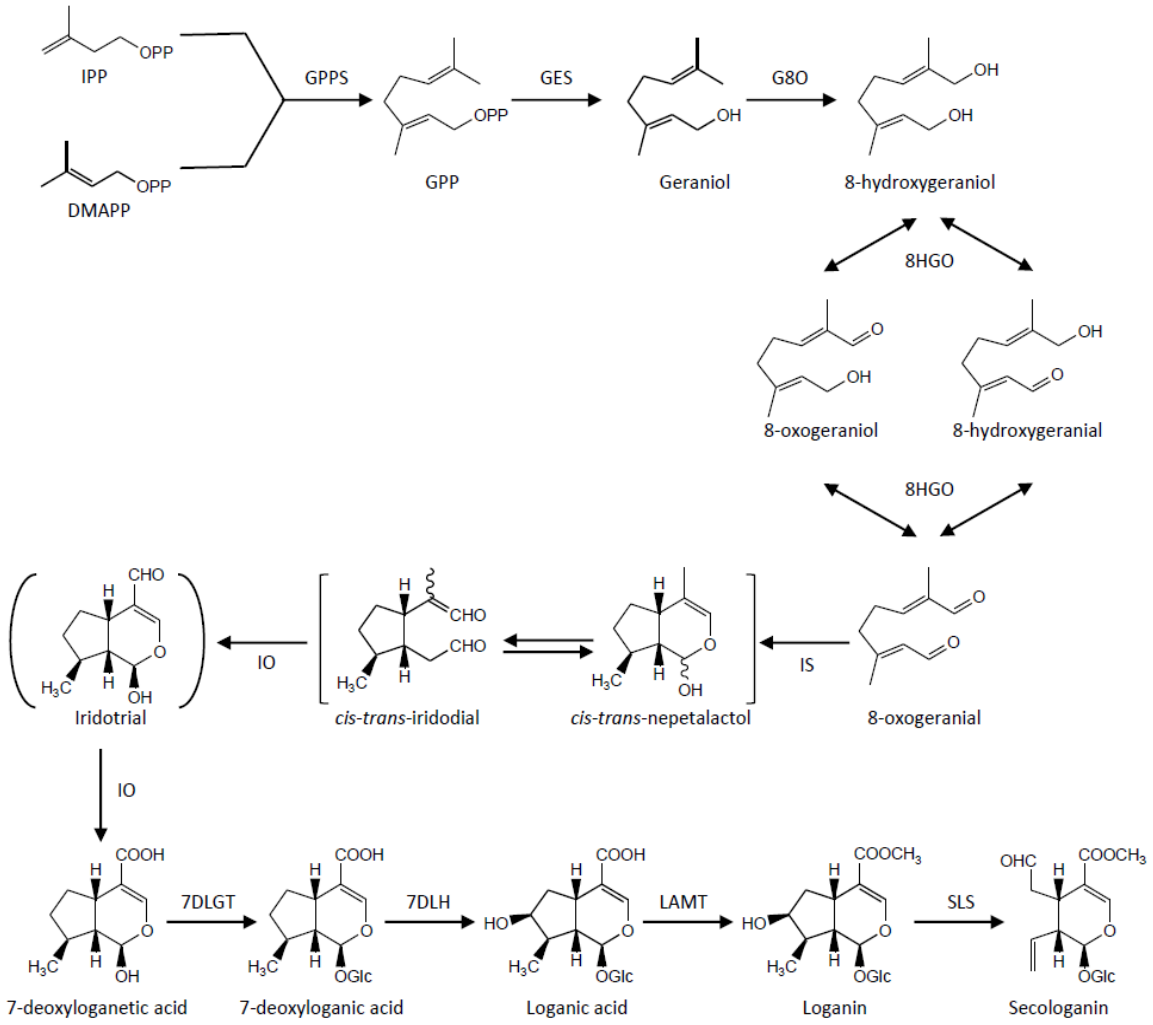

Fig. 4 Secoiridoid pathway of *Catharanthus roseus* leading to the terpenoid indole alkaloid precursor secologanin (Miettinen et al. 2014). 7-DLGT: 7-deoxyloganetic acid glucosyl transferase, 7-DLH: 7-deoxyloganic acid hydroxylase, 8-HGO: 8-hydroxygeraniol oxidoreductase, DMAPP: dimethylallyl diphosphate, G8O: geraniol 8-oxidase, GES: geraniol synthase, GPP: geranyl diphosphate, GPPS: geranyl diphosphate synthase, IO: iridoid oxidase, IPP: isopentenyl diphosphate, IS: iridoid synthase, LAMT: loganic acid *O*-methyltransferase, SLS: secologanin synthase.

Subcellular localization and cellular compartments of TIA biosynthesis

Biosynthesis of TIA involves intra- and intercellular compartments. Studies on the subcellular localization of enzymes in the MEP pathway to secoiridoids and of the TIA pathways have shown that at least four different subcellular compartments are involved, including plastid, cytosol, nucleus, and vacuole (Guirimand et al. 2010; Guirimand et al. 2011a; Guirimand et al. 2011b). **Figure 5** shows the different subcellular localization of the TIA enzymes in *C. roseus*.

The MEP pathway leading to geraniol is localized in the plastids (Mahroug et al. 2007). Geraniol is then transported across the plastid and stromules to the endoplasmic reticulum (ER), where the next enzyme G8O (G10H) is localized (Guirimand et al. 2009; Simkin et al. 2013). A series of enzymes for conversion of 8-hydroxygeraniol (10-hydroxygeraniol) to loganic acid is shown to be localized in the cytosol (IS) (Geu-Flores et al. 2012), both the cytosol and nucleus (8-HGO and 7-DLGT), and the ER (IO and 7-DLH) (Miettinen et al. 2014). LAMT forming loganin is localized in the cytosol, whereas SLS which catalyzed the formation of secologanin is anchored to the cytosolic face of the ER membranes (Guirimand et al. 2011a).

Tryptophan is derived from the shikimate pathway in the plastid and it has to move out to the cytosol, where TDC is mainly operated to yield tryptamine (De Luca and Cutler 1987). STR was shown to be localized in the vacuole. Therefore, both secologanin and tryptamine need to be transported to the vacuole to become available to STR (Mahroug et al. 2007; Guirimand et al. 2010). Subsequently, strictosidine is transported out of the vacuole to be deglucosylated by SGD which is associated with the nucleus (Guirimand et al. 2010). While the TIA pathway towards catharanthine is uncharacterized, the subcellular localization of enzymes for vindoline pathway is quite well studied. T16H, a cytochrome P450 is anchored to the ER membrane and OMT is found to homodimerize in the cytosol to facilitate the uptake of the T16H conversion product (Guirimand et al. 2011b). NMT is localized within the thylakoid membrane of chloroplast (De Luca and Cutler 1987; Dethier and De Luca 1993), whereas D4H and DAT were shown to operate as monomers that reside in both cytosol and nucleus (Guirimand et al. 2011b). Vindoline and catharanthine must be transported to the vacuole as CrPrx1, which mediates their coupling to produce AVLB, is localized in the vacuole (Costa et al. 2008). It is thought that also the further steps leading to VLB and VCR occur in the vacuole.

Besides the complexity at the subcellular level with enzymes being active in different subcellular compartments, the TIA pathway genes are also expressed in different cell types in the leaves, thus suggesting intercellular translocation of intermediates in TIA biosynthethic pathway (**Fig. 6**). The genes involved in the MEP and the early iridoid pathway until loganic acid are expressed in the internal phloem associated parenchyma (IPAP) cells of leaves (Burlat et al. 2004; Simkin et al. 2013; Miettinen et al. 2014). The genes involved in the biosynthesis of the indole precursor tryptamine (*TDC*), and the terpenoid precursor secologanin (*LAMT* and *SLS*), and TIA intermediates until at least 16-hydroxytabersonine-16-

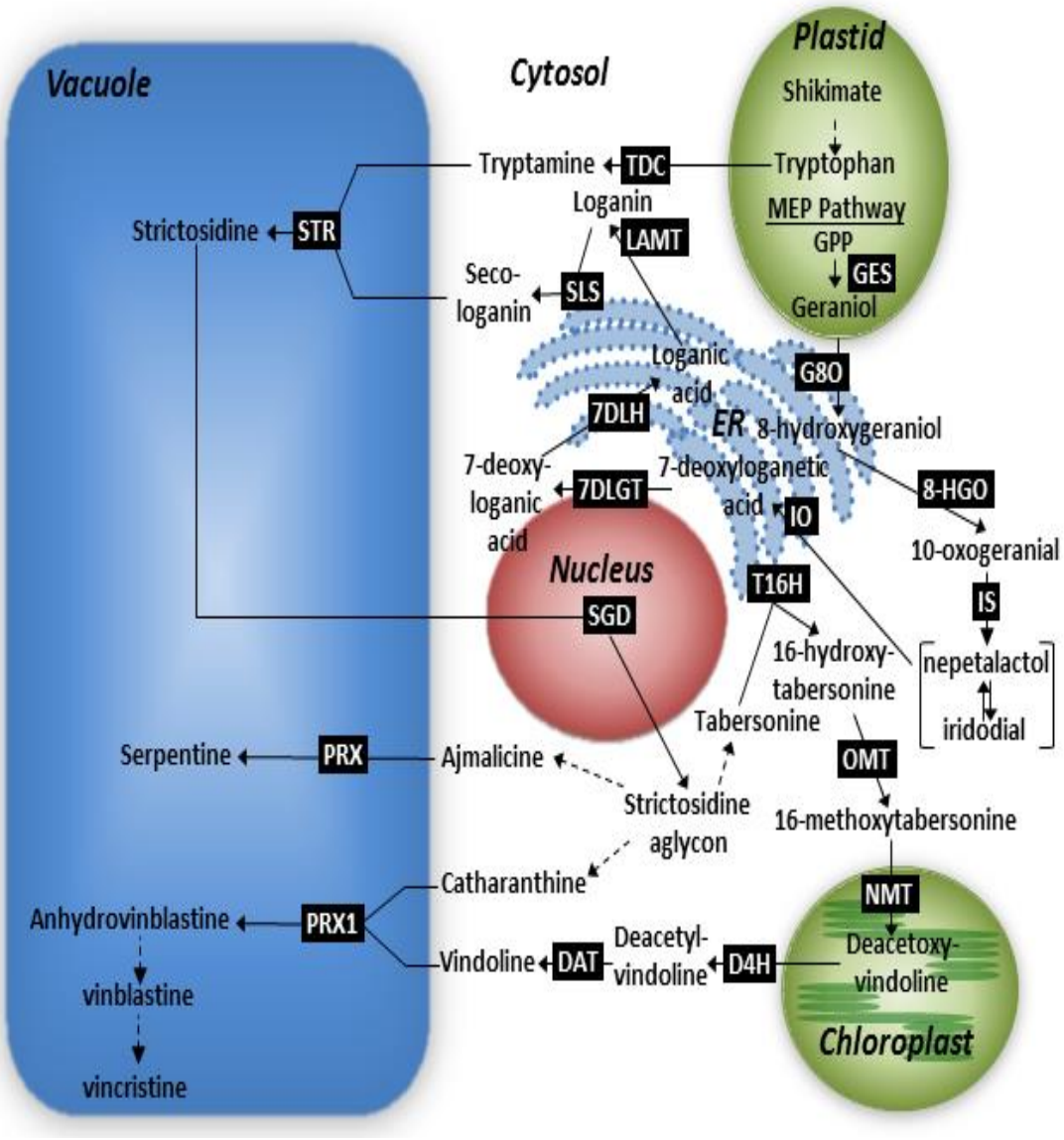

Fig. 5 Scheme of the subcellular localization of enzymes in TIA pathway of *Catharanthus roseus*. In a *Catharanthus* plant, different parts of the pathway are expressed in different cell types. ER: endoplasmatic reticulum, TDC: tryptophan decarboxylase, GPP: geranyl diphosphate, GES: geraniol synthase, G8O: geraniol 8-oxidase, 8-HGO: 8-hydroxygeraniol oxidoreductase, IS: iridoid synthase, IO: iridoid oxidase, 7DLGT: 7-deoxyloganetic acid glucosyl transferase, 7DLH: 7-deoxyloganic acid hydroxylase, LAMT: loganic acid *O*-methyltransferase, SLS: secologanin synthase, STR: strictosidine synthase, SGD: strictosidine *β*-D-glucosidase, T16H: tabersonine 16-hydroxylase, OMT: 16-hydoxytabersonine 16-*O*-methyltransferase; NMT: *N*-methyltransferase, D4H: desacetoxyvindoline 4-hydroxylase, DAT: deacetylvindoline 4-*O*-acetyltransferase; PRX: peroxidase, PRX1: peroxidase 1.

O-methyltransferase are expressed in the epidermal cells. In addition, secretion and accumulation of catharanthine in the leaf wax surface suggest that catharanthine biosynthesis in the leaf takes place in the epidermis cells (Roepke et al. 2010). An *N*-methyltransferase enzyme is associated with the thylakoid membrane of the chloroplasts, thus suggesting it is localized in the mesophyll cells which are rich in chloroplasts (De Luca and Cutler 1987; Dethier and De Luca 1993; Murata and De Luca 2005). The last two steps of vindoline biosynthesis occur in specialized cells laticifer and idioblast cells of aerial tissues (St-Pierre et al. 1999; Verma et al. 2012; Salim and De Luca 2013). In underground tissues, *TDC*, *STR*, and *MAT* mRNAs were found to be associated with the protoderm and cortical cells around the apical meristem of the root tip (St-Pierre et al. 1999; Laflamme et al. 2001). Neither *D4H* nor *DAT* transcripts nor gene products were ever detected in roots (St-Pierre et al. 1999), which is consistent with the accumulation of vindoline in the above-ground tissues only (Facchini and De Luca 2008).

Biosynthesis of C5 units for terpenoid precursors

Terpenoids form the largest group of plant natural products with about 40,000 compounds (Bohlmann and Keeling 2008). Terpenoids can be classified based on the number of isoprene units in the compound, e.g. hemiterpenoids (1 isoprene unit, C5), monoterpenoids (2 isoprene units, C10), sesquiterpenoids (3 isoprene units, C15), diterpenoids (4 isoprene units, C20), triterpenoids (6 isoprene units, C30), tetraterpenoids (8 isoprene units, C40), and polyterpenoids (a long chain of many isoprene units). All terpenoids including the secoiridoid precursor of TIA (secologanin) originate from the two C5 building blocks, i.e. IPP and its isomer DMAPP. These universal precursors of terpenoids are synthesized via two distinct metabolic routes, i.e. the mevalonate pathway and the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway (Rohmer 1999). In animals and fungi, the biosynthesis of terpenoids occurs through the mevalonate pathway, whereas the MEP pathway is prevalent for most of the prokaryotes. In plants, both the mevalonate pathway and the MEP pathway co-exist to produce a broad range of metabolites that are important for the plant growth and their interaction with environment (Rohmer 2007).

In the MEP pathway (**Fig. 7**), the biosynthesis of terpenoid precursors starts with glyceraldehyde 3-phosphate and pyruvate to produce 1-deoxy-D-xylulose 5-phosphate (DXP) by the enzyme 1-deoxy-D-xylulose 5-phosphate synthase (DXS). DXP is then reduced and isomerized to produce 2-*C*-methyl-D-erythritol 4-phosphate (MEP) by DXP reductoisomerase (DXR). This intermediate is converted in a series of steps to form IPP and

DMAPP. In the mevalonate pathway, IPP is derived from three molecules of acetyl-CoA that form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by HMG-CoA synthase (HMGS). Reduction of this compound by HMG-CoA reductase (HMGR) produces mevalonate. Mevalonate is phosphorylated to form mevalonate phosphate and mevalonate diphosphate by mevalonate kinase (MVK) and phosphomevalonate kinase (PMK), respectively. This intermediate is then decarboxylated by 5-diphosphomevalonate decarboxylase (MVD) to produce IPP. Isomerization of IPP to DMAPP is catalyzed by isopentenyl diphosphate isomerase (IDI) (Ramos-Valdivia et al. 1997). While the MEP pathway is a plastidial biosynthethic pathway as all enzymes in this pathway are localized in the plastid (Joyard et al. 2009), the mevalonate pathway is regarded as a cytosolic pathway as the localization of

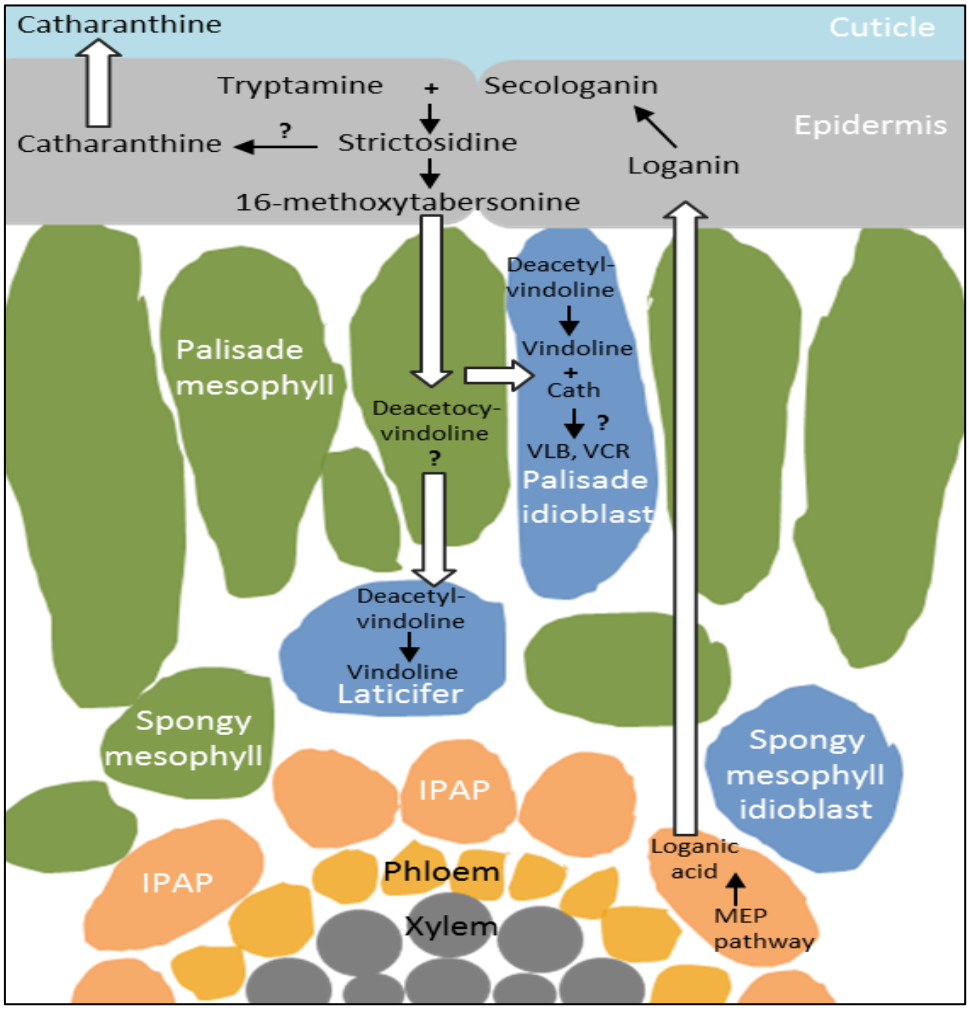

Fig. 6 Compartmentation of the TIA pathway in different cell types of the *Catharanthus roseus* leaf (adapted from Facchini and De Luca 2008). IPAP: internal phloem associated parenchyma, Cath: catharanthine, VLB: vinblastine, VCR: vincristine.

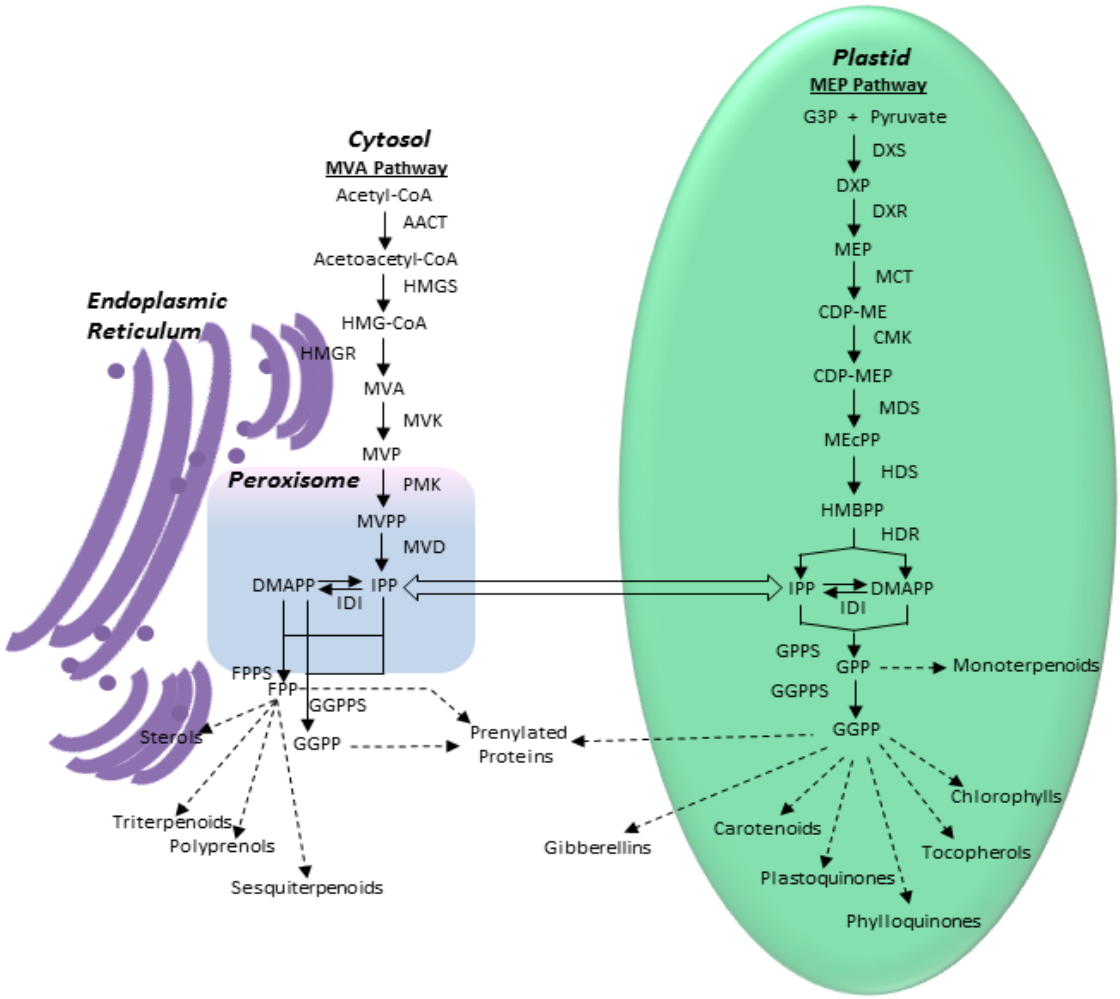

Fig. 7 The different groups of terpenoids are synthesized via two distinct metabolic routes, i.e. the MVA pathway and the MEP pathway (adapted from Pulido et al. 2012). Dashed arrows indicate multiple steps and open arrow represent transport of metabolites between subcellular compartments. AACT: acetoacetyl-CoA thiolase, CDP-ME: 4-(cytidine 5'-diphospho)-2-*C*-methyl-D-erythritol, CDP-MEP: CDP-ME 2-phosphate, CMK: CDP-ME kinase, DMAPP: dimethylallyl diphosphate, DXP: 1-deoxy-D-xylulose 5-phosphate, DXR: DXP reducto isomerase, DXS: DXP synthase, FPP: farnesyl diphosphate, FPPS: FPP synthase, G3P: glyceraldehyde 3-phosphate, GGPP: geranylgeranyl diphosphate, GGPPS: GGPP synthase, GPP: geranyl diphosphate, GPPS: GPP synthase, HMBPP: 1-hydroxy-2-methyl-2-butenyl 4-diphosphate, HDR: HMBPP reductase, HDS: HMBPP synthase, HMG-CoA: 3-hydroxy-3-methylglutaryl CoA, HMGR: HMG-CoA reductase, HMGS: HMG-CoA synthase, IDI: IPP isomerase, IPP: isopentenyl diphosphate, MCT: MEP cytidyltransferase, MDS: MEcPP synthase, MEcPP: ME 2,4-cyclodiphosphate, MEP: 2-*C*-methyl-D-erythritol 4-phosphate, MVA: mevalonic acid, MVD: 5-diphosphomevalonate decarboxylase, MVK: mevalonate kinase, MVP: 5-phosphomevalonate, MVPP: 5-diphosphomevalonate, PMK: 5-phosphomevalonate kinase.

the early biosynthetic steps are in the cytosol (Simkin et al. 2011). However, recent studies gave new insights on the subcellular distribution of the mevalonate pathway, as it seems that the mevalonate pathway is operating in the cytosol (HMGS and MVK), endoplasmic reticulum (HMGR), and peroxisomes (PMK, MVD, and IDI) (Reumann et al. 2007; Sapir-Mir et al. 2008; Simkin et al. 2011; Pulido et al. 2012).

The mevalonate and MEP pathways are localized in different subcellular compartments, each leading to a distinct set of terpenoid derivatives. The head to tail condensation of DMAPP and IPP generates geranyl diphosphate (GPP; C10), farnesyl diphosphate (FPP; C30), and geranylgeranyl diphosphate (GGPP; C20) by the GPP synthase (GPPS), FPP synthase, and GGPP synthase (GGPPS), respectively. These prenyltransferase including IDI are distributed in cytosol, plastid, mitochondria, and peroxisome (Sapir-Mir et al. 2008; Simkin et al. 2011; Thabet et al. 2011, Guirimand et al. 2012; Rai et al. 2013, Lange et al. 2013). However, the biosynthesis of FPP which serves as a precursor for sesquiterpenoids (C15), triterpenoids and sterols (C30) occurs primarily in the cytosolic/peroxisomal mevalonate pathway, whereas the plastidial MEP pathway provides GPP and GGPP units for the assembly of monoterpenoids (C10), diterpenoids (C20), tetraterpenoids and carotenoids (C40) (Tholl 2006).

Cross-talk between mevalonate pathway and MEP pathway

Ramos-Valdivia et al. (1997) in reviewing the early feeding experiments in terpenoid biosynthesis mentioned that in several studies different labeling percentages in the different C5-parts of various terpenoids were reported suggesting an exchange of one or both intermediates between the cellular compartments responsible for the mevalonate and MEP pathways (Ramos-Valdivia et al. 1997). Further, more recent studies also suggest that there is a cross-talk of isoprene precursors between the two pathways (Vranová et al. 2012). Feeding experiments using labeled 1-deoxy-D-xylulose (MEP pathway) or mevalonolactone (mevalonate pathway) have shown that the intermediates can be directed to a certain extent into the biosynthesis of phytosterols (Arigoni et al. 1997) and lutein (Schuhr et al. 2003), respectively.

Moreover, the cross-talk between these two pathways has been studied by blocking a specific step in the MEP and mevalonate pathway using chemical inhibitors or mutagenesis. Hemmerlin et al. (2003) showed that feeding labeled 1-deoxy-D-xylulose to tobacco BY-2 cells could partially rescue the inhibition of the mevalonate pathway by a HMGR-specific inhibitor mevinolin, and the sterols which normally derive from mevalonate were synthesized

via the MEP pathway. In addition, feeding mevalonate to tobacco BY-2 cells in the presence of a DXP-specific inhibitor fosmidomycin could overcome the growth inhibition by fosmidomycin resulting in mevalonate incorporation into plastoquinone, a product of the MEP pathway. Kasahara et al. (2002) also demonstrated that both MVA and MEP pathways can contribute to the biosynthesis of gibberellins and campesterol in *Arabidopsis* seedlings and the phenotypic defects caused by the block of the MVA and MEP pathways were partially rescued by exogenous application of the MEP and MVA precursors, respectively. These results suggest that the transport is possible in both directions. Laule et al. (2003) also showed an interaction between the cytosolic mevalonate and the plastidial MEP pathway by studying the levels of several metabolites and gene transcriptions after adding specific inhibitors of the respective pathways in *Arabidopsis thaliana* seedlings. However, their results suggest that the cross-flow of isopentenyl precursors between both pathways may occur in a unidirectional process, i.e. from plastidial MEP pathway to cytosolic mevalonate pathway and not vice versa. This result was supported by Dudareva et al. (2005) and Hampel et al. (2005) who also showed that the trafficking of IPP occurs unidirectionally from the plastids to cytosol. It was suggested that plastid membranes possess a unidirectional proton symport system for the export of specific isoprenoid intermediates involved in the metabolic cross-talk between cytosolic and plastidial pathways (Bick and Lange 2003).

IPP and short prenyl diphosphates (DMAPP, GPP, and FPP) are likely to be the intermediates that participate in the metabolic cross-flow between the MVA and MEP branches of the isoprenoid pathway network because 1) IPP and short prenyl diphosphates are substrates of enzymes in isoprenoid network branches connected to both the MVA and the MEP branches; 2) IPP and short prenyl diphosphates can be translocated through the plastid membrane that separates the MVA and MEP branches; 3) higher prenyl diphosphates, such as GGPP (C20), are not transported with appreciable efficiency through the plastid membrane (Bick and Lange 2003; Vranová et al. 2012).

Interestingly, the cross-talk of prenyl intermediates does not only affect biosynthesis directly, they also play a role by regulatory actions. For example, it was shown that the MEP pathway derived geranylgeranyl moiety plays an essential role in protein prenylation in the cytosol (Gerber et al. 2009), while MVA pathway derived farnesyl groups are employed for prenylation of proteins that have a regulatory effect on the MEP pathway by the activation of some of the MEP pathway genes (Courdavault et al. 2005a; 2005b).

Thesis Aims and Research Objectives

The research described in this thesis was conducted to study the channeling and regulation of the plant cell biosynthetic machinery in TIA biosynthesis in a broader sense in order to develop new synthetic biology approaches to improve the flux towards the terpenoid indole alkaloids (TIA) in *Catharanthus roseus* cell cultures. Several studies were carried out to investigate the metabolic effect and TIA accumulation in *C. roseus* cell suspension cultures upon precursor feeding, elicitation, and overexpression of the native *C. roseus* geraniol synthase in the plastid and cytosol. In these particular studies, the specific objectives are:

1. to analyze different terpenoid groups, i.e. monoterpenoid (TIA), triterpenoid (sterols), and tetraterpenoid (carotenoids) in different *C. roseus* cell lines, which may compete for the five carbon terpenoid precursors.
2. to evaluate metabolic changes and distribution of five carbon terpenoid precursors among the three indicated terpenoid groups in *C. roseus* cell suspension culture upon jasmonic acid elicitation which is employed to specifically induce TIA associated genes and increase flow through the TIA pathway.
3. to evaluate the metabolic changes due to mevalonic acid feeding as means to interfere in potential cross-talk and reduce potential outflow of MEP pathway intermediates to cytosolic non-TIA routes.
4. to study the production of TIA and precursors in *C. roseus* cell suspension culture upon geraniol feeding including the combination with jasmonic acid elicitation to determine potential precursor and transport limitations.
5. to engineer and overexpress *C. roseus* geraniol synthase in *C. roseus* suspension cells in different subcellular compartments (plastid and cytosol) to offer a constitutive solution for precursor availability while overcoming intracellular logistic restrictions and to evaluate the metabolic changes in these transformed cell suspension cultures.

Thesis Outline

Chapter 1 presents a general introduction and literature review of the studies on *C. roseus* alkaloid biosynthesis. **Chapter 2** reports the analysis of metabolites in the terpenoid pathway of *C. roseus* cell suspension cultures. Terpenoid indole alkaloids (monoterpenoid; C10), sterols (triterpenoid; C30), and carotenoids (tetraterpenoid; C40) were analyzed in nine *C. roseus* cell lines. Principal component analysis (PCA) was applied to distinguish the *C. roseus* cell lines based on their metabolite levels. The transcript levels of selected genes from

terpenoid pathways were also analyzed by q-PCR. The experiment described in **Chapter 3** explores the effect of jasmonic acid elicitation on the accumulation of monoterpenoid TIA and iridoid precursors (C10), sterols (C30), and carotenoids (C40) in a *C. roseus* cell suspension culture (cell-line CRPP) using liquid and gas chromatography. In addition, an NMR-based metabolomics approach was applied to analyze metabolomic changes in a broader context. The effect of mevalonic acid feeding on monoterpenoid (TIA and iridoid precursors), triterpenoid (sterol), and tetraterpenoid (carotenoid) production in a *C. roseus* cell suspension culture (cell-line CRPP) is reported in **Chapter 4**. **Chapter 5** describes the effect of geraniol feeding alone and in combination with jasmonic acid elicitation on the production of TIA and iridoid precursors in a *C. roseus* cell suspension culture (cell-line CRPP). **Chapter 6** describes the development of transgenic *C. roseus* cell suspension cultures overexpressing geraniol synthase in the plastid or cytosol. Furthermore, metabolic changes in the transformed *C. roseus* cells were investigated. **Chapter 7** summarizes the thesis and discusses future perspectives.

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