Terpenoids and terpenoid indole alkaloids in *Catharanthus roseus* cell suspension cultures

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To my mother who sacrifices
and my wife who compromises

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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.

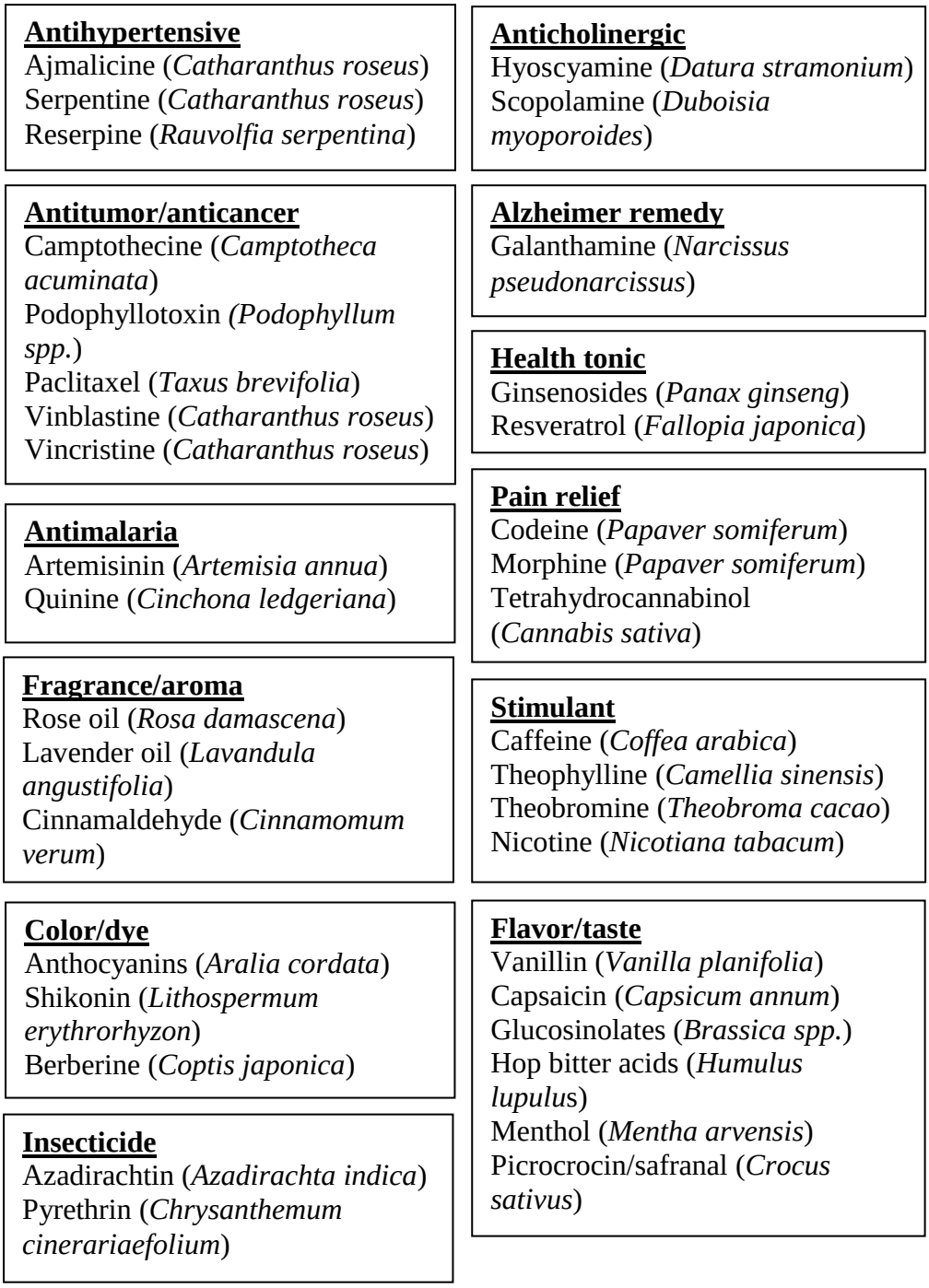

Fig. 1 Examples of plant secondary metabolites used for different purposes and the plant species from which they can be extracted.

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).

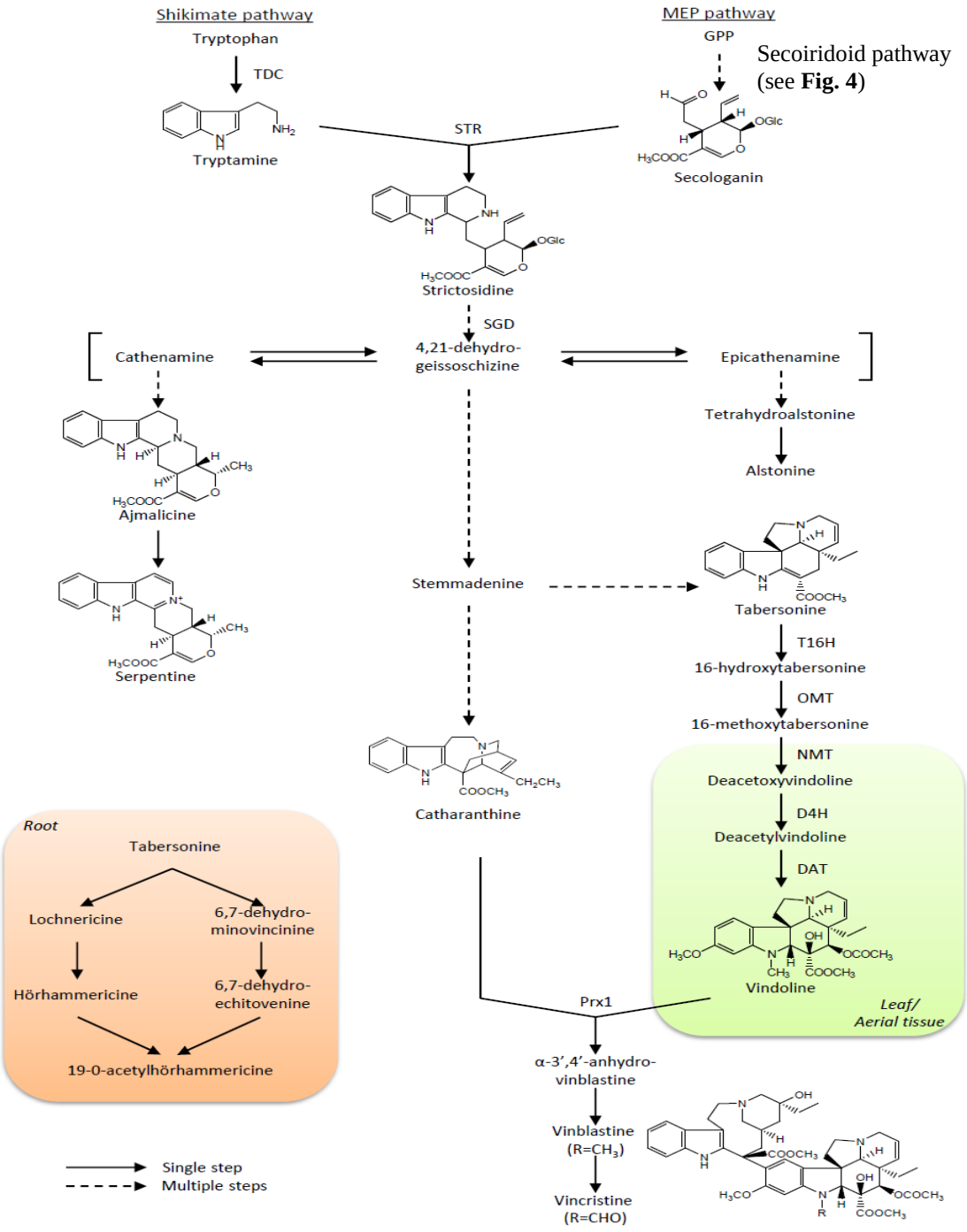

Fig. 3 Biosynthetic pathway of terpenoid indole alkaloids in *Catharanthus roseus* leading to the anticancer compounds vinblastine and vincristine. D4H: desacetoxyvindoline 4-hydroxylase, DAT: deacetylvindoline 4-*O*-acetyltransferase, GPP: geranyl diphosphate, NMT: *N*-methyltransferase, OMT: 16-hydroxytabersonine 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).

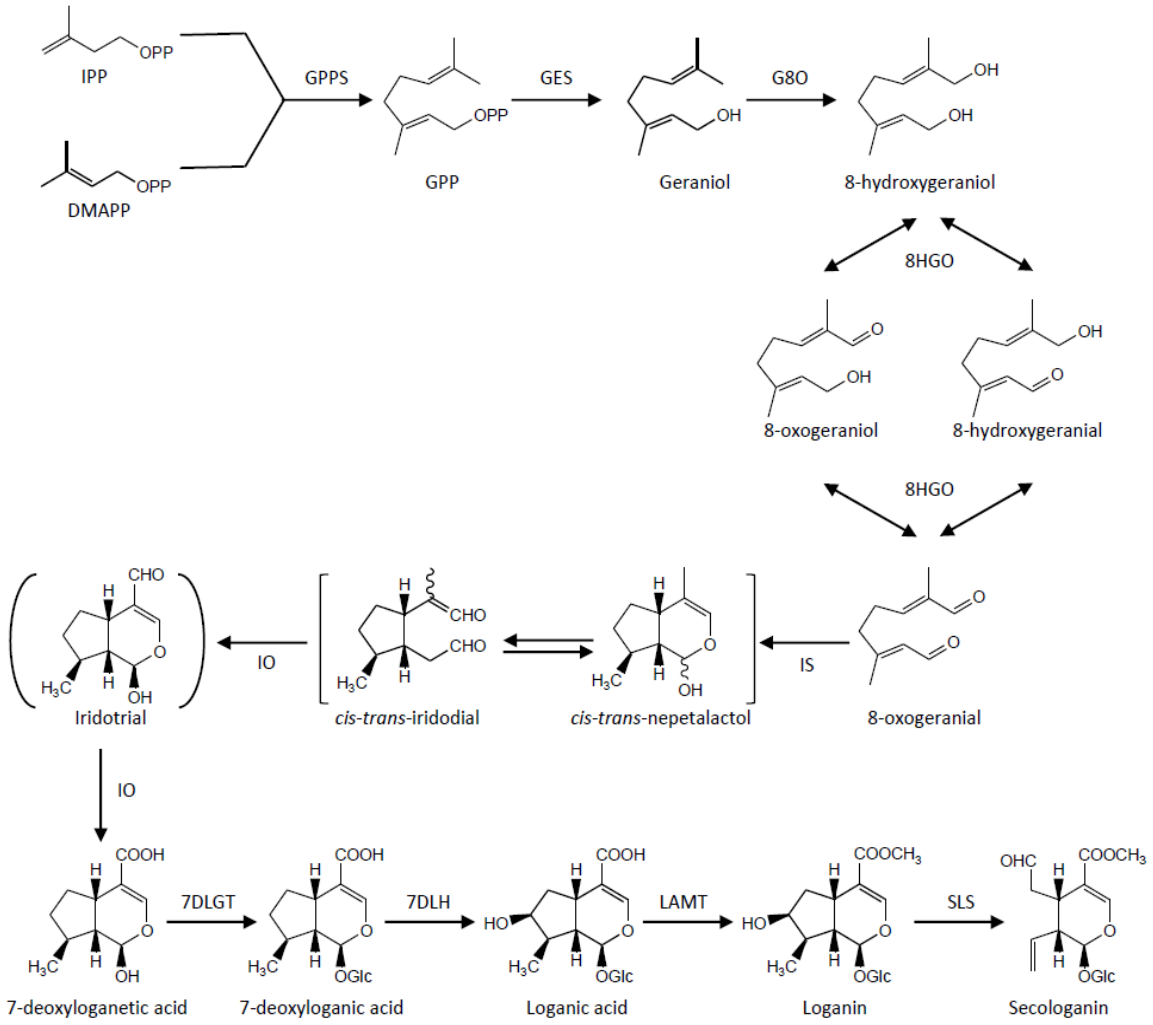

Fig. 4 Secoiridoid pathway of *Catharanthus roseus* leading to the terpenoid indole alkaloid precursor secologanin (Miettinen et al. 2014). 7-DLGT: 7-deoxyloganetic acid glucosyl transferase, 7-DLH: 7-deoxyloganic acid hydroxylase, 8-HGO: 8-hydroxygeraniol oxidoreductase, DMAPP: dimethylallyl diphosphate, 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-

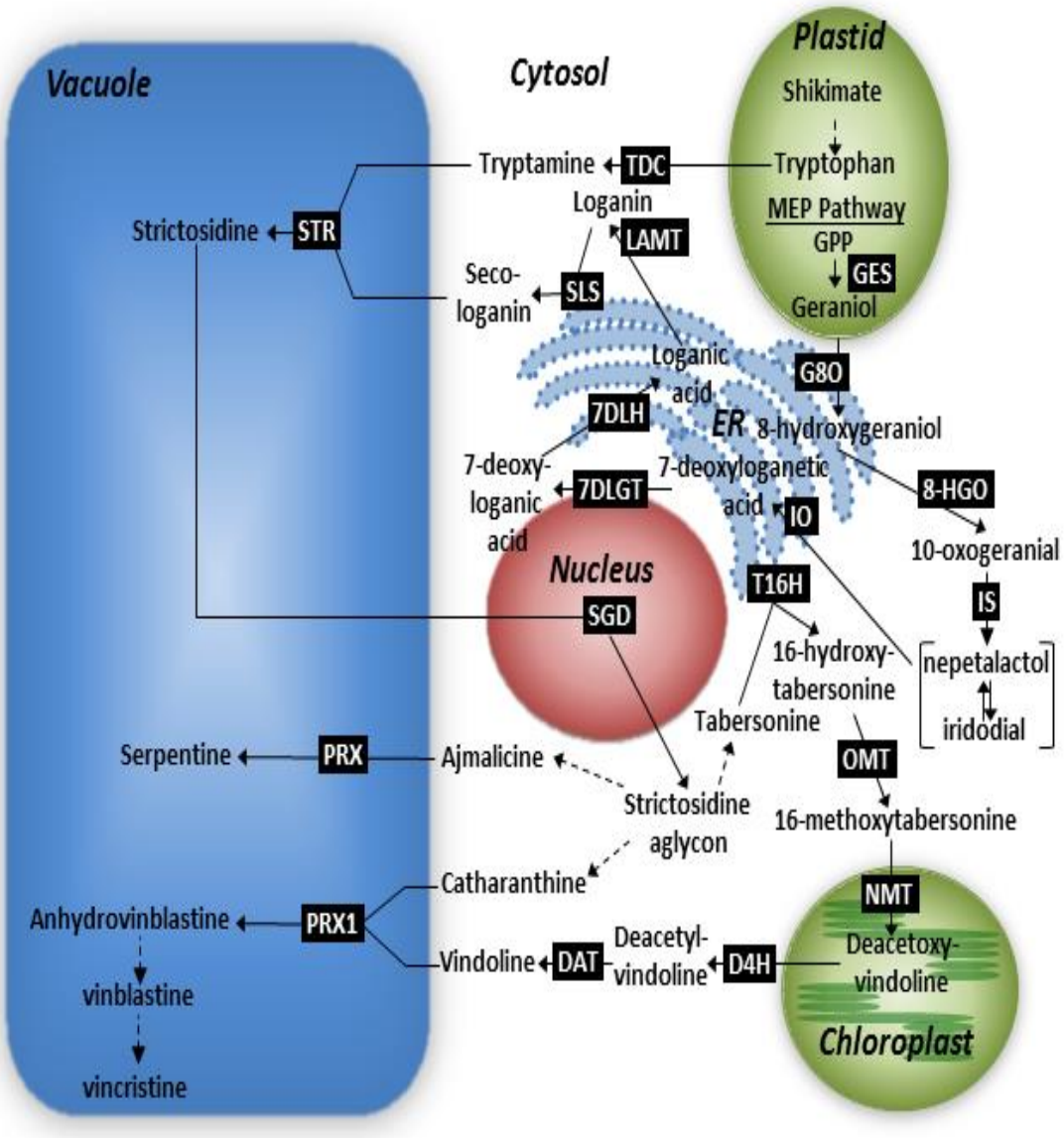

Fig. 5 Scheme of the subcellular localization of enzymes in TIA pathway of *Catharanthus roseus*. In a *Catharanthus* plant, different parts of the pathway are expressed in different cell types. ER: endoplasmatic reticulum, TDC: tryptophan decarboxylase, GPP: geranyl diphosphate, GES: geraniol synthase, 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

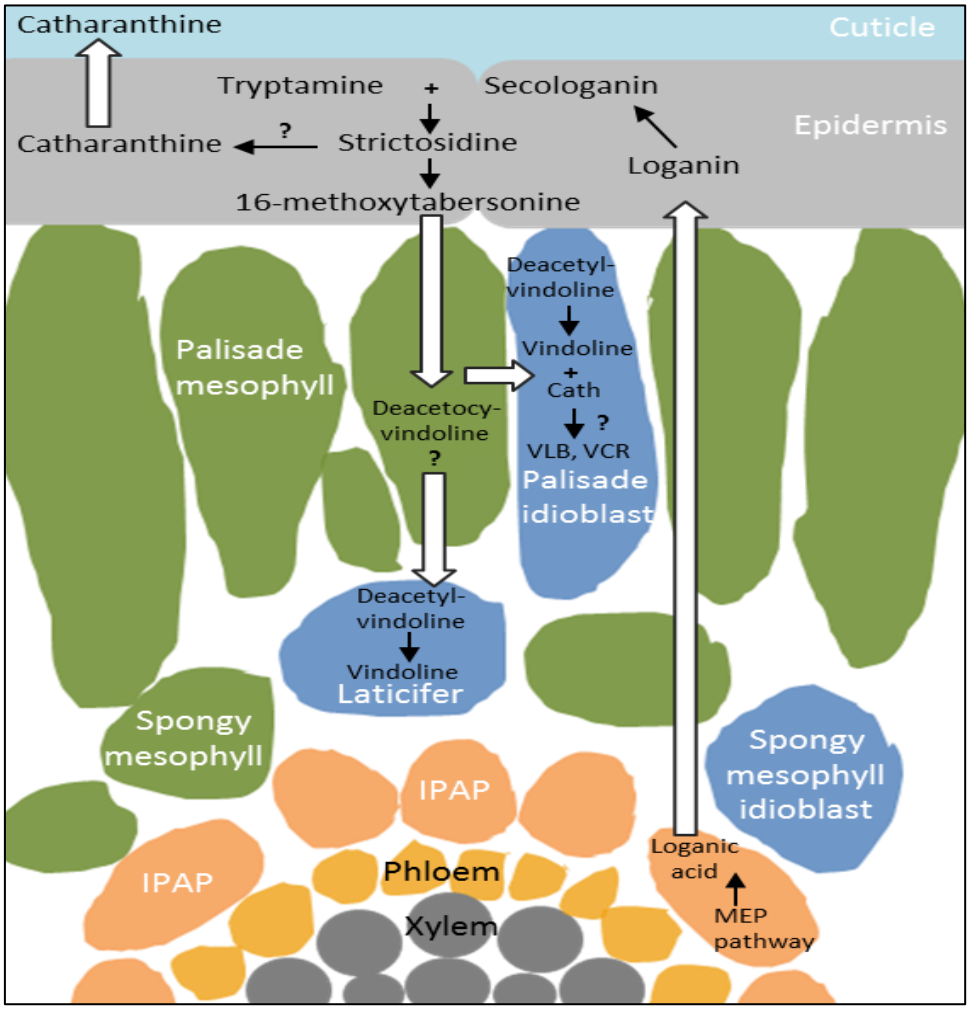

Fig. 6 Compartmentation of the TIA pathway in different cell types of the *Catharanthus roseus* leaf (adapted from Facchini and De Luca 2008). IPAP: internal phloem associated parenchyma, Cath: catharanthine, VLB: vinblastine, VCR: vincristine.

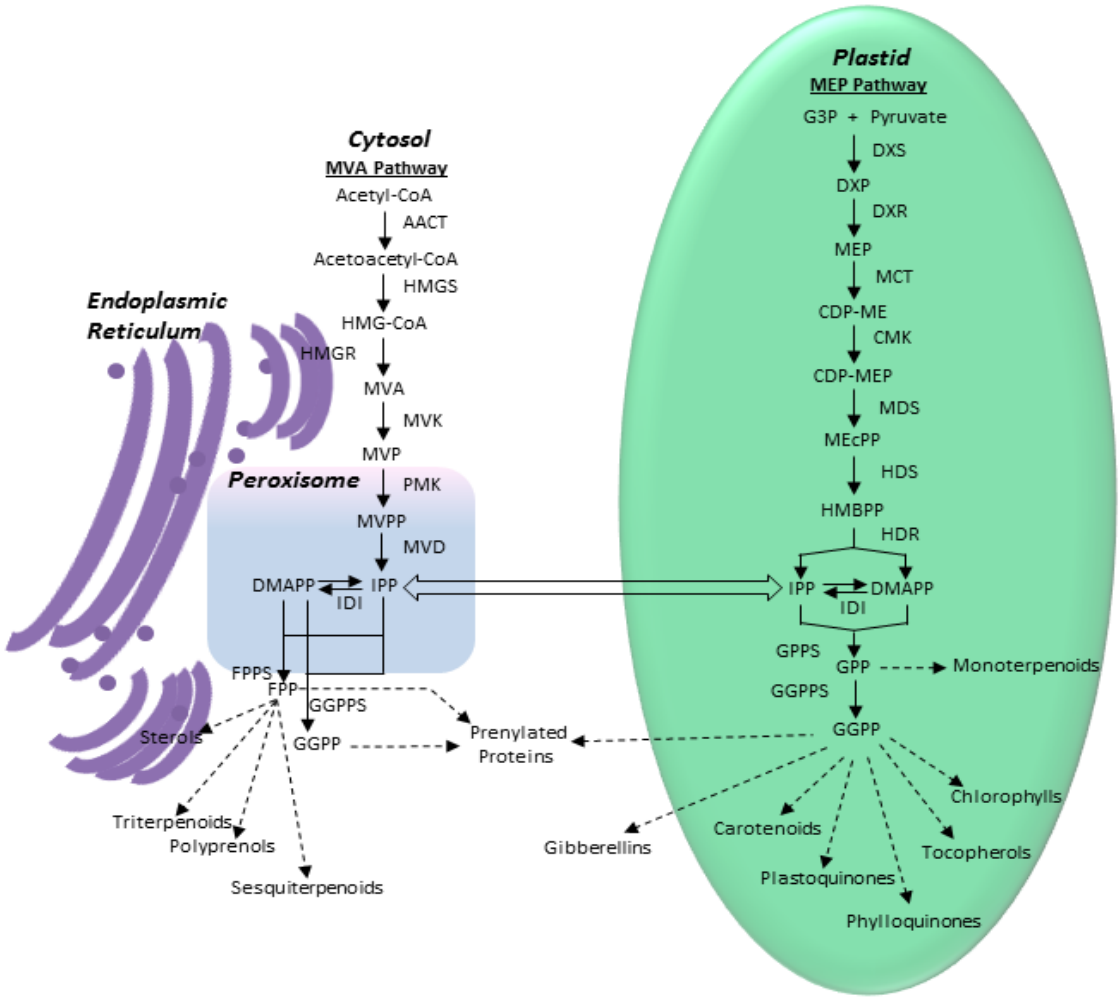

Fig. 7 The different groups of terpenoids are synthesized via two distinct metabolic routes, i.e. the MVA pathway and the MEP pathway (adapted from Pulido et al. 2012). Dashed arrows indicate multiple steps and open arrow represent transport of metabolites between subcellular compartments. AACT: acetoacetyl-CoA 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.

References

Alfermann AW, Peterson M (1995) Natural product formation by plant cell biotechnology – Results and perspectives. Plant Cell Tiss Organ Cult 43: 199–205

Arigoni D, Sagner S, Latzel C, Eisenreich W, Bacher A, Zenk MH (1997) Terpenoid biosynthesis from 1-deoxy-D-xylulose in higher plants by intramolecular skeletal rearrangement. Proc Natl Acad Sci USA 94: 10600–10605

Aslam J, Khan SH, Siddiqui ZH, Fatima Z, Maqsood M, Bhat MA, Nasim SA, Ilah A, Ahmad IZ, Khan SA, Mujib A, Sharma MP (2010) *Catharanthus roseus* (L.) G. Don. An important drug: it's applications and production. Int J Compr Pharm 1: 1–16

Bick JA, Lange BM (2003) Metabolic cross-talk between cytosolic and plastidial pathways of isoprenoid biosynthesis: unidirectional transport of intermediates across the chloroplast envelope membrane. Arch Biochem Biophys 415: 146–154

Blom TJM, Sierra M, van Vliet TB, Franke-van Dijk MEI, de Koning P, van Iren F, Verpoorte R, Libbenga KR (1991) Uptake and accumulation of ajmalicine into isolated vacuoles of cultured cells of *Catharanthus roseus* (L.) G. Don. and its conversion into serpentine. Planta 183: 170–177

Bohlmann J, Keeling CI (2008) Terpenoid biomaterials. Plant J 54: 656–669

Burlat V, Oudin A, Courtois M, Rideau M, St-Pierre B (2004) Co-expression of three MEP pathway genes and geraniol 10-hydroxylase in internal phloem parenchyma of *Catharanthus roseus* implicates multicellular translocation of intermediates during the biosynthesis of monoterpene indole alkaloids and isoprenoid-derived primary metabolites. Plant J 38: 131–141

Choi YE, Kim YS, Paek KY (2008) Types and designs of bioreactors for hairy root culture. In: Dutta Gupta S, Ibaraki Y (eds.) Plant Tissue Culture Engineering, Series: Focus on Biotechnology, Springer, Dordrecht, pp. 161–171

Courdavault V, Burlat V, St-Pierre B, Giglioli-Guivarc'h N (2005a) Characterisation of CaaX-prenyltransferases in *Catharanthus roseus*: relationships with the expression of genes involved in the early stages of monoterpenoid biosynthetic pathway. Plant Sci 168: 1097–1107

Courdavault V, Thiersault M, Courtois M, Gantet P, Oudin A, Doireau P, St-Pierre B, Giglioli-Guivarc'h N (2005b) CaaX-prenyltransferases are essential for expression of genes involved in the early stages of monoterpenoid biosynthetic pathway in *Catharanthus roseus* cells. Plant Mol Biol 57: 855–870

Contin A, van der Heijden R, Lefeber AW, Verpoorte R (1998) The iridoid glucoside secologanin is derived from the novel triose phosphate/pyruvate pathway in a *Catharanthus roseus* cell culture. FEBS Lett 434: 413–416

Contin A, van der Heijden, ten Hoopen HJG, Verpoorte R (1999) Effects of alkaloid precursor feeding and elicitation on the accumulation of secologanin in a *Catharanthus roseus* cell suspension culture. Plant Cell Tiss Organ Cult 56: 111–119

Costa MMR, Hilliou F, Duarte P, Pereira LG, Almeida I, Leech M, Memelink J, Barcelo AR, Sottomayor M (2008) Molecular cloning and characterization of a vacuolar class III peroxidase involved in the metabolism of anticancer alkaloids in *Catharanthus roseus*. Plant Physiol 146: 403–417

De Carolis E, Chan F, Balsevich J, De Luca V (1990) Isolation and characterization of a 2-oxoglutarate dependent dioxygenase involved in the second-to-last step in vindoline biosynthesis. Plant Physiol 94: 1323–1329

De Carolis E, De Luca V (1993) Purification, characterization and kinetic analysis of a 2-oxoglutarate-dependent dioxygenase involved in vindoline biosynthesis from *Catharanthus roseus*. J Biol Chem 268: 5504–5511

De Luca V, Cutler AJ (1987) Subcellular localization of enzymes involved in indole alkaloid biosynthesis in *Catharanthus roseus.* Plant Physiol 85: 1099–1102

De Luca V, Balsevich J, Tyler RT, Kurz WG (1987) Characterization of a novel *N*-methyltransferase (NMT) from *Catharanthus roseus* plants: Detection of NMT and other enzymes of the indole alkaloid biosynthetic pathway in different cell suspension culture systems. Plant Cell Rep 6: 458–461

De Luca V, Marineau C, Brisson N (1989) Molecular cloning and analysis of cDNA encoding a plant tryptophan decarboxylase: comparison with animal dopa decraboxylases. Proc Natl Acad Sci USA 86: 2582–2586

De Waal A, Meijer AH, Verpoorte R (1995) Strictosidine synthase from *Catharanthus roseus*: purification and characterization of multiple forms. Biochem J 306: 571–580

Dethier M, De Luca V (1993) Partial purification of a *N*-methyltransferase involved in vindoline biosynthesis in *Catharanthus roseus*. Phytochemistry 32:673–678

Dixon RA (2001) Natural products and plant disease resistance. Nature 411: 843–847

Döller G, Alfermann AW, Reinhard E (1976) Production of indole alkaloids in tissue cultures of *Catharanthus roseus*. Planta Med 30: 14–20

Ducos JP, Terrier B, Courtois D, Pétiard V (2008) Improvement of plastic-based disposable bioreactors for plant science needs. Phytochem Rev 7: 607–613

Dudareva N, Andersson S, Orlova I, Gatto N, Reichelt M, Rhodes D, Boland W, Gershenzon J (2005) The nonmevalonate pathway supports both monoterpene and sesquiterpene formation in snapdragon flowers. Proc Natl Acad Sci USA 102: 933–938

El-Sayed MA, Verpoorte R (2002) Effect of phytohormones on growth and alkaloid accumulation by a *Catharanthus roseus* cell suspension culture fed with the alkaloid precursors tryptamine and loganin. Plant Cell Tiss Organ Cult 68: 265– 270

El-Sayed M, Verpoorte R (2007) *Catharanthus* terpenoid indole alkaloids: biosynthesis and regulation. Phytochem Rev 6:277–305

Eibl R, Eibl D (2008) Design of bioreactors suitable for plant cell and tissue cultures. Phytochem Rev 7: 593–598

Facchini PJ, De Luca V (2008) Opium poppy and Madagascar periwinkle: Model non-model systems to investigate alkaloid biosynthesis in plants. Plant J 54: 763–784

Fujita Y, Hara Y, Suga C, Morimoto T (1981) Production of shikonin derivatives by cell suspension cultures of *Lithospermum erythrorhizon*. Plant Cell Rep 1: 61–63

Fujita Y (1988) Industrial production of shikonin and berberine. In: Bock G, Hansh J (eds.) Applications of Plant Cell and Tissue Culture. John Wiley and Sons, Chichester, pp. 228–238

Geerlings A, Ibañez MM, Memelink J, van der Heijden R, Verpoorte R (2000) Molecular cloning and analysis of strictosidine beta-D-glucosidase, an enzyme in terpenoid indole alkaloid biosynthesis in *Catharanthus roseus*. J Biol Chem 275: 3051– 3056

Georgiev MI, Weber J, Maciuk A (2009) Bioprocessing of plant cell cultures for mass production of targeted compounds. Appl Microbiol Biotechnol 83: 809–823

Gerber E, Hemmerlin A Hartmann M, Heintz D, Hartmann MA, Mutterer J, Rodríguez-Concepció M, Boronat A, van Dorsselaer A, Rohmer M, Crowell DN, Bach TJ (2009) The plastidial 2-*C*-methyl-D-erythritol 4-phosphate pathway provides the isoprenyl moiety for protein geranylgeranylation in tobacco BY-2 cells. Plant Cell 21: 285–300

Gertlowski C, Petersen M (1993) Influence of the carbon source on growth and rosmarinic acid production in suspension cultures of *Coleus blumei*. Plant Cell Tiss Organ Cult 34: 183–190

Geu-Flores F, Sherden NH, Courdavault V, Burlat V, Glenn WS, Wu C, Nims E, Cui Y, O'Connor SE (2012) An alternative route to cyclic terpenes by reductive cyclization in iridoid biosynthesis. Nature 492: 138–142

Guarnaccia R, Botta L, Coscia CJ (1974) Biosynthesis of acidic iridoid monoterpene glucosides in *Vinca rosea*. J Am Chem Soc 96: 7079–7084

Guirimand G, Burlat V, Oudin A, Lanoue A, St-Pierre B, Courdavault V (2009) Optimization of the transient transformation of *Catharanthus roseus* cells by particle bombardment and its application to the subcellular localization of hydroxymethylbutenyl 4-diphosphate synthase and geraniol 10-hydroxylase. Plant Cell Rep 28: 1215–1234

Guirimand G, Courdavault V, Lanoue A, Mahroug S, Guihur A, Blanc N, Giglioli-Guivarc'h N, St-Pierre B, Burlat V (2010) Strictosidine activation in Apocynaceae: towards a "nuclear time bomb"? BMC Plant Biol 10: 182–201

Guirimand G, Guihur A, Ginis O, Poutrain P, Héricourt F, Oudin A, Lanoue A, St-Pierre B, Burlat V, Courdavault V (2011a) The subcellular organization of strictosidine biosynthesis in *Catharanthus roseus* epidermis highlights several trans-tonoplast translocations of intermediate metabolites. FEBS J 278: 749-763

Guirimand G, Guihur A, Poutrain P, Héricourt F, Mahroug S, St-Pierre B, Burlat V, Courdavault V (2011b) Spatial organization of the vindoline biosynthetic pathway in *Catharanthus roseus*. J Plant Physiol 168: 549–557

Guirimand G, Guihur A, Phillips MA, Oudin A, Glévarec G, Melin C, Papon N, Clastre M, St-Pierre B, Manuel Rodríguez-Concepción M, Burlat V, Courdavault V (2012) A single gene encodes isopentenyl diphosphate isomerase isoforms targeted to plastids, mitochondria and peroxisomes in *Catharanthus roseus*. Plant Mol Biol 79: 443–459

Hampel D, Mosandl A, Wüst M (2005) Biosynthesis of mono- and sesquiterpenes in carrot roots and leaves (*Daucus carota* L.): metabolic cross-talk of cytosolic mevalonate and plastidial methylerythritol phosphate pathways. Phytochemistry 66: 305–311

Hemmerlin A, Hoeffler JH, Meyer O, Tritsch D, Kagan IA, Grosdemange-Billiard C, Rohmer M, Bach TJ (2003) Cross-talk between the cytosolic mevalonate and the plastidial methylerythritol phosphate pathways in Tobacco Bright Yellow-2 cells. J Biol Chem 18: 26666–26676

Hong SB, Peebles CAM, Shanks JV, San KY, Gibson SI (2006) Expression of the *Arabidopsis* feedback-insensitive anthranilate synthase holoenzyme and tryptophan decarboxylase genes in *Catharanthus roseus* hairy roots. J Biotechnol 122: 28–38

Irmler S, Schröder G, St-Pierre B, Crouch NP, Hotze M, Schmidt J, Strack D, Matern U, Schröder J (2000) Indole alkaloid biosynthesis in *Catharanthus roseus*: new enzyme activities and identification of cytochrome P450 CYP72A1 as secologanin synthase. Plant J 24: 797–804

Joyard J, Ferro M, Masselon C, Seigneurin-Berny D, Salvi D, Garin J, Rolland N (2009) Chloroplast proteomics and the compartmentation of plastidial isoprenoid biosynthetic pathways. Mol Plant 2: 1154–1180

Kasahara H, Hanada A, Kuzuyama T, Takagi M, Kamiya Y, Yamaguchi S (2002) Contribution of the mevalonate and methylerythritol phosphate pathways to the biosynthesis of gibberellins in *Arabidopsis*. J Biol Chem 277: 45188–45194

Kim JH, Yun JH, Hwang YS, Byun SY, Kim DI (1995) Production of taxol and related taxanes in *Taxus brevifolia* cell cultures: effect of sugar. Biotechnol Lett 17: 101–106

Krueger RJ, Carew DP (1978) *Catharanthus roseus* tissue culture: the effects of precursors on growth and alkaloid production. J Nat Prod 41: 327–331

Knobloch KH, Berlin J (1980) Influence of medium composition on the formation of secondary compounds in cell suspension cultures of *Catharanthus roseus* (L.) G. Don. Z Naturforsch 35c: 551–556

Laflamme P, St-Pierre B, De Luca V (2001) Molecular and biochemical analysis of a Madagascar periwinkle root-specific minovincinine-19-hydroxy-*O*-acetyltransferase. Plant Physiol 125: 189–198

Lange BM, Ahkami A (2013) Metabolic engineering of plant monoterpenes, sesquiterpenes and diterpenes – current status and future opportunities. Plant Biotechnol J 11: 169–196

Laule O, Fürholz A, Chang H-S, Zhu T, Wang X, Heifetz PB, Gruissem W, Lange BM (2003) Cross-talk between cytosolic and plastidial pathways of isoprenoid biosynthesis in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 100: 6866–6871

Lee-Parsons CWT, Royce AJ (2006) Precursor limitations in methyl jasmonate-induced *Catharanthus roseus* cell cultures. Plant Cell Rep 25: 607–612

Luijendijk TJC, van der Meijden E, Verpoorte R (1996) Involvement of strictosidine as a defensive chemical in *Catharanthus roseus*. J Chem Ecol 22: 1355–1366

Luijendijk TJC, Stevens LH, Verpoorte R (1998) Purification and characterization of strictosidine beta-D-glucosidase from *Catharanthus roseus* cell suspension cultures. Plant Physiol Biochem 36: 419–425

Madyastha KM, Coscia CJ (1979) Detergent-solubilized NADPH-cytochrome *c* (P-450) reductase from the higher plant, *Catharanthus roseus*. Purification and characterization. J Biol Chem 254: 2419–2427

Mahroug S, Burlat V, St-Pierre B (2007) Cellular and subcellular organisation of the monoterpenoid indole alkaloid pathway in *Catharanthus roseus*. Phytochem Rev 6: 363– 381

Meijer AH, Lopes Cardoso MI, Voskuilen JT, De Waal A, Verpoorte R, Hoge JH (1993) Isolation and characterization of a cDNA clone from *Catharanthus roseus* encoding NADPH:cytochrome P-450 reductase, an enzyme essential for reactions catalysed by cytochrome P-450 mono-oxygenases in plants. Plant J 4: 47–60

Memelink J, Kijne JW, van der Heijden R, Verpoorte R (2001) Genetic modification of plant secondary metabolite pathways using transcriptional regulators. Adv Biochem Eng Biotechnol 72: 103–125

Merillon JM, Doireau P, Guillot A (1986) Indole alkaloid accumulation and tryptophan decarboxylase activity in *Catharanthus roseus* cells cultured in three different media. Plant Cell Rep 5: 23–26

Miettinen K, Dong L, Navrot N, Schneider T, Burlat V, Pollier J, Woittiez L, van der Krol S, Lugan R, Ilc T, Verpoorte R, Oksman-Caldentey KM, Martinoia E, Bouwmeester H, Goossens A, Memelink J, Werck-Reichhart D (2014) The seco-iridoid pathway from *Catharanthus roseus*. Nat Comm. p. 3606. doi:10.1038/ncomms4606

Misawa M, Goodbody AE (1996) Production of antitumor compounds by plant cell cultures. In: DiCosmo F, Misawa M (eds.) Plant cell culture secondary metabolism toward industrial application. CRC press, Boca Raton, pp. 123–138

Mizukami H, Nordlov H, Lee SL, Scott AI (1979) Purification and properties of strictosidine synthase (an enzyme condensing tryptamine and secologanin) from *Catharanthus roseus* cultured cells. Biochemistry 18: 3760–3763

Moreno PRH, van der Heijden R, Verpoorte R (1993) Effect of terpenoid precursor feeding and elicitation on formation of indole alkaloids in cell suspension cultures of *Catharanthus roseus*. Plant Cell Rep 12: 702–705

Moreno PRH, van der Heijden R, Verpoorte R (1995) Cell and tissue cultures of *Catharanthus roseus*: A literature survey II. Updating from 1988 to 1993. Plant Cell Tiss Organ Cult 42: 1–25

Morgan JA, Shanks JV (2000) Determination of metabolic rate-limitations by precursor feeding in *Catharanthus roseus* hairy root cultures. J Biotechnol 79: 137–145

Mujib A, Ilah A, Aslam J, Fatima S, Siddiqui ZH, Maqsood M (2012) *Catharanthus roseus* alkaloids: application of biotechnology for improving yield. Plant Growth Regul 68: 111–127

Murata J, De Luca V (2005) Localization of tabersonine 16-hydroxylase and 16-OH-tabersonine-16-*O*-methyltransferase to leaf epidermal cells defines them as a major site of precursor biosynthesis in the vindoline pathway in *Catharanthus roseus*. Plant J 44: 581−594

Murata J, Roepke J, Gordon H, De Luca V (2008) The leaf epidermome of *Catharanthus roseus* reveals its biochemical specialization. Plant Cell 20: 524–542

Namdeo AG (2007) Plant cell elicitation for production of secondary metabolites: A review. Pharmacogn Rev 1: 69–79

Noble RL (1990) The discovery of the vinca alkaloids – chemotherapeutic agents against cancer. Biochem Cell Biol 68: 1344-1351

Noble RL, Beer CT, Cutts JH (1958) Role of chance observation in chemotherapy: *Vinca rosea*. Ann NY Acad Sci 76: 882–894

Pan Q (2014) Metabolomic characteristics of *Catharanthus roseus* plants in time and space. PhD Thesis, Leiden University, The Netherlands. Chapter 2, Terpenoid indole alkaloids biosynthesis and its regulation in *Catharanthus roseus*: a literature review from genes to metabolome, pp. 55–74. ISBN 9789462035584

Pennings EJM, Groen BW, Duine JA, Verpoorte R (1989) Tryptophan decarboxylase from *Catharanthus roseus* is a pyridoxoquinoprotein. FEBS Lett 255: 97–100

Pfitzner U, Zenk MH (1989) Homogeneous strictosidine synthase isoenzymes from cell suspension cultures of *Catharanthus roseus*. Planta Med 55: 525–530

Power R, Kurz WGW, De Luca V (1990) Purification and characterization of acetylcoenzyme A: deacetylvindoline 4-*O*-acetyltransferase from *Catharanthus roseus*. Arch Biochem Biophys 279: 370–376

Pulido P, Perello C, Rodriguez-Concepcion M (2012) New insights into plant isoprenoid metabolism. Mol Plant 5: 964–967

Ramawat KG (2007) Secondary plant products in nature. In: Ramawat KG, Merillon JM (eds.) Biotechnology, Secondary Metabolites, Plants and Microbes, Science Publishers, Enfield, pp. 1–20

Rai A, Smita SS, Sing AK, Shanker K, Nagegowda DA (2013) Heteromeric and homomeric geranyl diphosphate synthase from *Catharanthus roseus* and their role in monoterpenoid indole alkaloid biosynthesis. Mol Plant 6: 1531–1549

Ramos-Valvidia AC, van der Heijden R, Verpoorte R (1997) Isopentenyl diphosphate isomerase: A core enzyme in isoprenoid biosynthesis. A review of its biochemistry and function. Nat Prod Rep 14: 591–603

Reumann S, Babujee L, Ma C, Wienkoop S, Siemsen T, Antonicelli GE, Rasche N, Lüder F, Weckwerth W, Jahn O (2007) Proteome analysis of *Arabidopsis* leaf peroxisomes reveals novel targeting peptides, metabolic pathways, and defense mechanisms. Plant Cell 19: 3170–3793

Rischer H, Oresic M, Seppänen-Laakso T, Katajamaa M, Lammertyn F, Ardiles-Diaz W, Van Montagu MCE, Inzé D, Oksman-Caldentey KM, Goossens A (2006) Gene-to-metabolite networks for terpenoid indole alkaloid biosynthesis in *Catharanthus roseus* cells. Proc Natl Acad Sci USA 103: 5614–5619

Roepke J, Salim V, Wu M, Thamm AMK, Murata J, Ploss K, Boland W, De Luca V (2010) *Vinca* drug components accumulate exclusively in leaf exudates of Madagascar periwinkle. Proc Natl Acad Sci USA 107: 15287–15292

Rohmer M (1999) The discovery of a mevalonate-independent pathway for isoprenoid biosynthesis in bacteria, algae and higher plants. Nat Prod Rep 16:565–574

Rohmer M (2007) Diversity in isoprene unit biosynthesis: The methylerythritol phosphate pathway in bacteria and plastids. Pure Appl Chem 79: 467–823

Saklani A, Kutty SK (2008) Plant-derived compounds in clinical trials. Drug Discov Today 13: 161–171

Salim V, De Luca V (2013) Towards complete elucidation of monoterpene indole alkaloid biosynthesis pathway: *Catharanthus roseus* as a pioneer system. In: Giglioli-Guivarc'h N (ed.) Advances of Botanical Research – New Light on Alkaloid Biosynthesis and Future Prospects. Academic Press, London, pp. 1–37

Sapir-Mir M, Mett A, Belausov E, Tal-Meshulam S, Frydman A, Gidoni D, Eyal Y (2008) Peroxisomal localization of *Arabidopsis* isopentenyl diphosphate isomerases suggests that part of the plant isoprenoid mevalonic acid pathway is compartmentalized to peroxisomes. Plant Physiol 148: 1219–1228

Sarin R (2005) Useful metabolites from plant tissue cultures. Biotechnol 4: 79–93

Sato F, Yamada Y (1984) High berberine producing cultured *Coptis japonica* cells. Phytochemistry 23: 281–285

Schuhr CA , Radykewicz1 T, Sagner S, Latzel C, Zenk MH, Arigoni D, Bacher A, Rohdich F, Eisenreich W (2003) Quantitative assessment of cross-talk between the two isoprenoid biosynthesis pathways in plants by NMR spectroscopy. Phytochem Rev 2: 3–16

Schröder G, Unterbusch E, Kaltenbach M, Schmidt J, Strack D, De Luca V, Schröder J (1999) Light-induced cytochrome P450-dependent enzyme in indole alkaloid biosynthesis: tabersonine 16-hydroxylase. FEBS Lett 458: 97–102

Simkin AJ, Guirimand G, Papon N, Courdavault V, Thabet I, Ginis O, Bouzid S, Giglioli-Guivarc'h N, Clastre M (2011). Peroxisomal localisation of the final steps of the mevalonic acid pathway in planta. Planta 234: 903–914

Simkin AJ, Miettinen K, Claudel P, Burlat V, Guirimand G, Courdavault V, Papon N, Meyer S, Godet S, St-Pierre B, Giglioli-Guivarc'h N, Fischer MJ, Memelink J, Clastre M (2013) Characterization of the plastidial geraniol synthase from Madagascar periwinkle which initiates the monoterpenoid branch of the alkaloid pathway in internal phloem associated parenchyma. Phytochemistry 85: 36–43

Sottomayor M, Lopez-Serrano M, DiCosmo F, Barcelo AR (1998) Purification and characterization of alpha-3',4'-anhydrovinblastine synthase (peroxidase-like) from *Catharanthus roseus* (L) G. Don. FEBS Lett 428: 299–303

Sottomayor M, Lopes Cardoso I, Pereira LG, Ros Barceló A (2004) Peroxidase and the biosynthesis of terpenoid indole alkaloids in the medicinal plant *Catharanthus roseus* (L.) G. Don. Phytochem Rev 3: 159–171

Stöckigt J, Husson H, Kan-Fan C, Zenk M (1977) Cathenamine, a central intermediate in the cell free biosynthesis of ajmalicine and related indole alkaloids. J Chem Soc Chem Commun 6: 164–166

St-Pierre B, De Luca V (1995) A cytochrome P-450 monooxygenase catalyzes the first step in the conversion of tabersonine to vindoline in *Catharanthus roseus*. Plant Physiol 109: 131–139

St-Pierre B, Vazquez-Flota FA, De Luca V (1999) Multicellular compartmentation of *Catharanthus roseus* alkaloid biosynthesis predicts intercellular translocation of a pathway intermediate. Plant Cell 11: 887–900

Svoboda GH, Johnson IS, Gorman M, Neuss N (1962) Current status of research on the alkaloids of *Vinca rosea* Linn. (*Catharanthus roseus* G. Don). J Pharm Sci 51: 707–720

Thabet I, Guirimand G, Courdavault V, Papon N, Godet S, Dutilleul C, Bouzid S, Giglioli-Guivarc'h N, Clastre M, Simkin AJ (2011) The subcellular localization of periwinkle farnesyl diphosphate synthase provides insight into the role of peroxisome in isoprenoid biosynthesis. J Plant Physiol 168: 2110–2116

Thanh NT, Murthy HN, Yu KW, Hahn EJ, Paek KY (2005) Methyl jasmonate elicitation enhanced synthesis of ginsenoside by cell suspension cultures of *Panax ginseng* in 5 l balloon type bubble bioreactors. Appl Microbiol Biotechnol 67: 197–201

Tholl D (2006) Terpene synthases and the regulation, diversity and biological roles of terpene metabolism. Curr Opin Plant Biol 9: 297–304

van der Heijden R, Verpoorte R, Ten Hoopen HJG (1989) Cell and tissue cultures of *Catharanthus roseus* (L.) G. Don: a literature survey. Plant Cell Tiss Organ Cult 18: 231-280

van der Heijden R, Jacobs DI, Snoeijer W, Hallard D, Verpoorte R (2004) The *Catharanthus* alkaloids: pharmacognosy and biotechnology. Curr Med Chem 11: 607– 628

Vázquez-Flota F, Hernández-Domínguez E, Miranda-Ham MdL, Monforte-González M (2009) A differential response to chemical elicitors in *Catharanthus roseus* in vitro cultures. Biotechnol Lett 31: 591–595

Verma P, Mathur AK, Srivastava A, Mathur A (2012) Emerging trends in research on spatial and temporal organization of terpenoid indole alkaloid pathway in *Catharanthus roseus:* a literature update. Protoplasma 249: 255-268

Verpoorte R, van der Heijden R, Memelink J (1998) Plant biotechnology and the production of alkaloids: prospects of metabolic engineering. In: GA Cordell (ed.) The Alkaloids, volume 50. Academic Press, San Diego, pp. 453–508

Verpoorte R, van der Heijden R, ten Hoopen HJG, Memelink J (1999) Metabolic engineering of plant secondary metabolite pathways for the production of fine chemicals. Biotechnol Lett 21: 467–479

Verpoorte R, van der Heijden R, Memelink J (2000) Engineering the plant cell factory for the secondary metabolite production. Transgenic Res 9: 323–343

Verpoorte R, Memelink J (2002) Engineering secondary metabolite production in plants. Curr Opin Biotechnol 13: 181–187

Verpoorte R, Contin A, Memelink J (2002) Biotechnology for the production of plant secondary metabolites. Phytochem Rev 1: 13–25

Verpoorte R, ten Hoopen HJG (2006) Plant cell biotechnology. In: Ratledge C, Kristiansen B (eds.) Basic Biotechnology, third edition. Cambridge University Press, Cambridge, pp. 549–557

Vranová E, Coman D, Gruissem W (2012) Structure and dynamics of the isoprenoid pathway network. Mol Plant 5: 318–333

Whitmer S, Canel C, Hallard D, Gonçalves C, Verpoorte R (1998) Influence of precursor availability on alkaloid accumulation by transgenic cell line of *Catharanthus roseus*. Plant Physiol 116: 853–857

Whitmer S, van der Heijden R, Verpoorte R (2002a) Effect of precursor feeding on alkaloid accumulation by a tryptophan decarboxylase overexpressing transgenic cell line T22 of *Catharanthus roseus*. J Biotechnol 96:193–203

Whitmer S, van der Heijden R, Verpoorte R (2002b) Effect of precursor feeding on alkaloid accumulation by a strictosidine synthase overexpressing transgenic cell line S1 of *Catharanthus roseus*. Plant Cell Tiss Organ Cult 69:85–93

Yamamoto H, Katano N, Ooi A, Inoue K (1999) Transformation of loganin and 7-deoxyloganin into secologanin by *Lonicera japonica* cell suspension cultures. Phytochemistry 50: 417–422

Yamamoto H, Sha M, Kitamura Y, Yamaguchi M, Katano N, Inoue K (2002) lridoid biosynthesis: 7-deoxyloganetic acid 1-*O*-glucosyltransferase in cultured *Lonicera japonica* cells. Plant Biotechnol 19: 295–301

Zenk MH, El-Shagi H, Arens H, Stockigt J, Weiler EW, Deus B (1977) Formation of the indole alkaloids serpentine and ajmalicine in cell suspension cultures of *Catharanthus roseus*. In: Barz W, Reinhard E, Zenk MH (eds.) Plant Tissue Culture and Its Biotechnological Application. Springer, Berlin, pp. 27–44

Zhao J, Verpoorte R (2007) Manipulating indole alkaloid production by *Catharanthus roseus* cell cultures in bioreactors: from biochemical processing to metabolic engineering. Phytochem Rev 6: 435–457

Zhou ML, Shao JR, Tang YX (2009) Production and metabolic engineering of terpenoid indole alkaloids in cell cultures of the medicinal plant *Catharanthus roseus* (L.) G. Don (Madagascar periwinkle). Biotechnol Appl Biochem 52: 313–323

Chapter 2

Analysis of metabolites in the terpenoid pathway of *Catharanthus roseus* cell suspensions

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Abstract

In *Catharanthus roseus* cell cultures, the monoterpenoid pathway has been shown to be a limiting factor in terpenoid indole alkaloid (TIA) production. This could be due to competition at the level of isopentenyl diphosphate::dimethylallyl diphosphate (C5) which leads to the biosynthesis of different terpenoid groups. For future engineering of the terpenoid pathway, chemical characterization of *C. roseus* cell cultures is a necessity. Therefore, in this study nine *C. roseus* cell suspension lines were characterized by analyzing the levels of the major terpenoids derived from different biosynthetic pathways which may compete for the same precursors; TIA (monoterpenoid, C10), carotenoids (tetraterpenoid, C40), and sterols (triterpenoid, C30). Among the cell lines, CRPP (S) was the most promising TIA-producing cell line which provided more TIA (24 µmol/g DW) than carotenoids (15 µmol/g DW) and sterols (2 µmol/g DW). However, when considering the distribution of the isopentenyl-precursor (C5), the carotenoids which assemble from 8 x C5 represent twofold more C5-units (122 µmol/g DW) than the TIA in this cell line. In the CRPP (G), A12A2 (G), and A12A2 (S) cell lines, the C5 distribution was predominant towards carotenoid biosynthesis as well, resulting in a relatively high accumulation of carotenoids. The geranylgeranyl diphosphate (C20) pathway towards carotenoid production is therefore considered competitive towards TIA biosynthesis. For channeling more precursors to the TIA, the branch point for C10 and C20 seems an interesting target for metabolic engineering. Using principal component analysis of the chromatographic data, we characterized the cell lines chemically based on their metabolite levels. The information on the metabolic composition of *C. roseus* cell cultures is useful for developing strategies to engineer the metabolic pathways and for selection of cell lines for future studies.

Introduction

Catharanthus roseus (Apocynaceae) produces more than 130 terpenoid indole alkaloids (TIA), including the antihypertensive monomeric alkaloids ajmalicine and serpentine, as well as some bisindole alkaloids, such as the anticancer agents vinblastine and vincristine (van der Heijden et al. 2004). Terpenoid indole alkaloids in *C. roseus* are biosynthesized from the central intermediate strictosidine, a product of condensation between the aldehyde function of the iridoid secologanin and the amino group of the indole tryptamine. Tryptamine derives from the shikimate pathway, whereas the iridoid moiety originates from 10-hydroxygeraniol (also known as 8-hydroxygeraniol) after hydroxylation of geraniol produced from geranyl diphosphate (GPP; C10) (El-Sayed and Verpoorte 2007;

Salim and De Luca 2013; **Fig. 1**). Geranyl diphosphate is synthesized from isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), which is subject to further condensation, thus generating farnesyl diphosphate (FPP; C15), and geranylgeranyl diphosphate (GGPP; C20). Farnesyl diphosphate leads to the formation of triterpenoids (sterols; C30) and sesquiterpenoids (C15), while GGPP leads to the production of diterpenoids (C20) and tetraterpenoids (carotenoids; C40) (Rohmer 1999; **Fig. 1**).

The two terpenoid building blocks, IPP and DMAPP can be produced via two distinct metabolic routes, i.e. the mevalonate (MVA) pathway in cytosol and the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway in plastids (Rohmer 1999). In plants, both MVA and MEP pathways coexist, while active in different subcellular compartments, each leading to a distinct set of terpenoid derivatives (**Fig. 1**). However, there is evidence of cross-talk between the IPP::DMAPP pools even though the MVA and MEP pathways are present in different cellular compartments in plant cells (Rohmer 1999; Hemmerlin et al. 2003; Schuhr et al. 2003; Laule et al. 2003). In addition, the MVA pathway has a regulatory effect on the MEP pathway by the activation of some of MEP pathway genes (Courdavault et al. 2005).

The generation of IPP::DMAPP and GPP and its distribution into different biosynthetic routes is of specific interest as they are precursors of the monoterpenoid geraniol, from which secologanin and the pharmaceutically important TIA are derived in *C. roseus*. The production levels of the bisindole alkaloids in *C. roseus* are low, which limits availability, resulting in high market prices (van der Heijden et al. 2004; Zhou et al. 2009). Plant cell cultures have been used as a potential continuous and reliable source of the bioactive metabolites (Moreno et al. 1995; van der Heijden et al. 2004; Pietrosiuk et al. 2007; Pati et al. 2010). Despite efforts to optimize growing and production conditions, large-scale cultivation of *C. roseus* cells for commercial production of TIA has not yet been obtained (Moreno et al. 1995; van der Heijden et al. 2004; Zhao and Verpoorte 2007).

Engineering of metabolic pathways has a great potential to enhance the metabolic fluxes into the TIA pathway, thus increasing feasibility for commercial production of TIA by plant cell and tissue cultures (Verpoorte et al. 2002; Zhao and Verpoorte 2007; Zhou et al. 2009; O'Connor 2012; Glenn et al. 2013). Based on feeding studies in *C. roseus* cell cultures, the terpenoid pathway has been shown to be a limiting factor in TIA production (Moreno et al. 1993; Whitmer et al. 2002; van der Heijden et al. 2004; Zhao and Verpoorte 2007). This could be due to a lack of precursors/intermediates, associated with low gene expression or enzyme activity in the MEP pathway, and/or it might be due to competition at the level of IPP::DMAPP and GPP with conversion steps favoring either geraniol and derived

monoterpenoids, versus GGPP and derived carotenoids and/or FPP and derived sterols. Chemical characterization of *C. roseus* cell cultures will provide important information on their metabolic composition and a better understanding of the possible changes induced during the experiments, both of which are prerequisite to engineer the metabolic pathway.

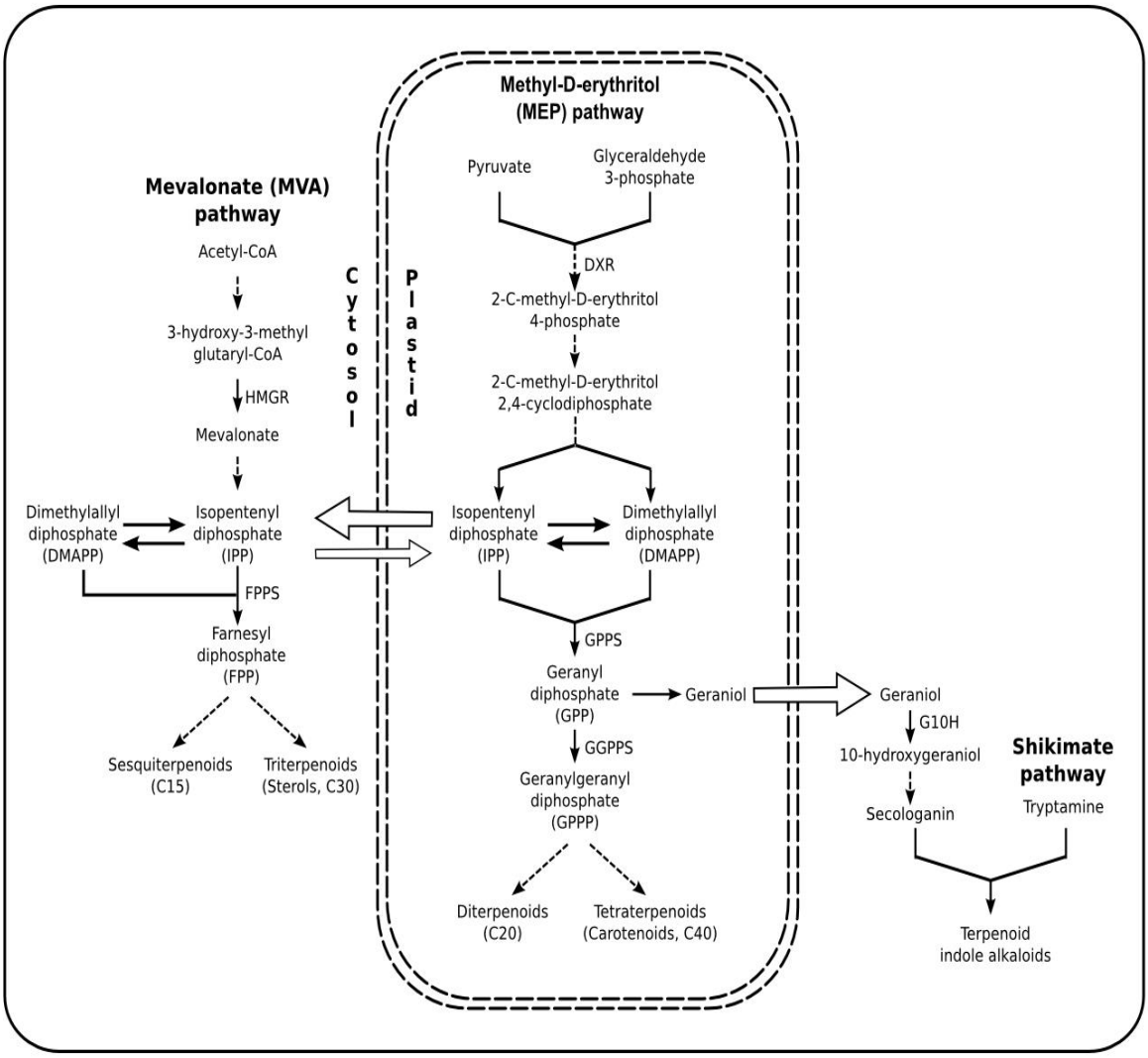

Fig. 1 The terpenoid biosynthetic pathway localized in cytosol and plastid generate different groups of terpenoid (adapted from Laule et. al 2003). Arrows with dashed lines indicate several biosynthetic steps. DXR: 1-deoxy-D-xylulose-5-phosphate reducto isomerase, FPPS: farnesyl diphosphate synthase, G10H: geraniol 10-hydroxylase (G8O: geraniol 8-oxidase), GPPS: geranyl diphosphate synthase, GGPPS: geranylgeranyl diphosphate synthase, HMGR: 3-hydroxy-3-methylglutaryl-CoA reductase.

Most characterization studies of *C. roseus* cell cultures have been focused on TIA production with little attention given to other metabolite groups in associated biosynthetic pathways that might compete for the same precursors. In this study, nine *C. roseus* cell suspension lines were analyzed for their production levels of TIA, iridoid and indole

precursors, as well as the sterols and carotenoids as the major representatives of triterpenoid and tetraterpenoid groups, respectively. Furthermore, the total accumulation of TIA, carotenoids, and sterols and the distribution of five-carbon (C5) precursors among the different terpenoid groups were evaluated. The metabolite data were also analyzed using principal component analysis (PCA) to determine which metabolites are responsible in discriminating the cell lines. In addition, the mRNA transcription levels of some representative genes of the associated terpenoid pathways were profiled.

The objective of the present study was to characterize several *C. roseus* cell lines for accumulation of major chemical groups derived from the different terpenoid biosynthetic pathways and to identify candidate lines with a high TIA-producing capacity for further in depth studies to enhance channeling of the C5 precursors towards the TIA.

Materials and methods

Plant cell cultures

The cell lines of *C. roseus* suspension cultures were developed in the past 20 years and maintained in the Natural Products Laboratory, Institute of Biology, Leiden University, The Netherlands. Nine cell lines used for this study were labeled as A12A2 (G), A12A2 (S), CRPP (G), CRPP (S), A11 (G), A11 (S), CRPM (G), CRPM (S), and CATL (S) (Mustafa et al. 2011). Some of them have been employed in the studies of terpenoid indole alkaloid, phenylpropanoid and salicylic acid pathways. The cell lines were grown in MS (Murashige and Skoog 1962) or B5 (Gamborg et al. 1968) medium supplemented with either 20 g/l glucose (G) or 30 g/l sucrose (S) in 250 ml Erlenmeyer flasks with 100 or 70 ml culture volume per flask. The cell cultures were maintained for more than ten years as batch suspension cultures subcultured every 7 – 21 days by taking various ratios of cells to fresh medium, depending on the line. Vitamin and growth regulator requirements varied with the lines (see **Table 1** for the cell line characteristics). Flasks were closed with T-32 silicon foam stoppers (Shin Etsu, Tokyo, Japan) and placed on a gyratory shaker (110 rpm) at 25 ± 1 ^{o}C under continuous light (10.8 – 27 µE/m^2/sec).

Table 1 Characteristics of *Catharanthus roseus* cell suspension lines employed in the study (Natural Products Laboratory, Institute of Biology,Leiden University, The Netherlands)

Cell Lines[a]	Medium[b]	Growth cycle (days)	Method Subculture	Phenotype	Description
A12A2 (G)	MS; with thiamine (1 mg/l), glucose (20 g/l), pH 5.8	7	1:1 dilution of 100 ml culture into 100 ml medium and divided to two flasks	Green cells, micro aggregates	• The A12A2 cell line derived from 9CR58 cell line, which was the habituated version of the original cell line 58CR. The parent culture was initiated from seeds in 1983. Then, the A12A2 cells have been maintained in Murashige and Skoog (MS) medium deprived from growth hormones and supplemented with 30 g/l sucrose (Moreno et al. 1993) or 20 g/l glucose. • The A12A2 cell line was used in several experiments and reported to accumulate tryptamine, strictosidine, ajmalicine (Moreno et al. 1993; El-Sayed and Verpoorte, 2002). • It is a high salicylic acid production cell line, which accumulate 2,3-dihydroxybenzoic acid after a fungal elicitation (Mustafa and Verpoorte 2007) and 2,5-dihydroxybenzoic acid glucoside after salicylic acid elicitation (Mustafa et al. 2009). • Collu et al. (2002) used A12A2 cell line to study the relationship between G10H (G8O) and alkaloid accumulation.
A12A2 (S)	MS; with thiamine (1 mg/l), sucrose (30 g/l), pH 5.8	14	Transfer 20 ml culture into 50 ml medium	Green cells, micro aggregates	
CRPP (G)	B5; with NAA (1.86 mg/l), glucose (20 g/l), pH 5.8	21	1:1 dilution of 100 ml culture into 100 ml medium and divided to two flasks	Dark green cells, micro aggregates	• The CRPP cell line was initiated in 1993 from *C. roseus* cv. Pacifica Punch. • It produced lower level of salicylic acid than the A12A2 cell line upon fungal elicitation.
CRPP (S)	B5; with NAA (1.86 mg/l), sucrose (30 g/l), pH 5.8	21	Transfer 20 ml culture into 50 ml medium	Dark green cells, micro aggregates	
A11 (G)	B5; with NAA (1.86 mg/l), glucose (20 g/l), pH 5.8	14	1:1 dilution of 100 ml culture into 100 ml medium and divided to two flasks	Green aggregates	• The A11 cell line derived from 9CR58 cell line, which was originated from 58CR cell line by changing to B5 medium supplemented with 1.86 mg/l NAA and 20 g/l sucrose. • A11 cell line had been used for studying the iridoid biosynthesis and reported to

A11 (S)	B5; with NAA (1.86 mg/l), sucrose (30 g/l), pH 5.8	14	Transfer 20 ml culture into 50 ml medium	Green-yellowish cells, micro aggregates	accumulate secologanin rather than tryptamine (Contin et al. 1999). • A11 cells produced G10H (G8O), strictosidine, and ajmalicine (5-, 10- and 2.5-fold, respectively) higher than the A12A2 cells (Contin et al. 1999).
CRPM (G)	MS; with thiamine (0.4 mg/l), no pyridoxine, no nicotinic acid, no glycine, NAA (2 mg/l), kinetin (0.2 mg/l), glucose (20 g/l), pH 5.8	7	1:1 dilution of 100 ml culture into 100 ml medium and divided to two flasks	Creamy fine cells	• The CRPM cell line derived from 58CR cell line. • It was used to study the G10H (G8O) activity (Collu et al. 2002) • It was a successful model for transformation of isochorismate synthase gene (Mustafa and Verpoorte 2007). • It produced a high number of vacuoles and was used as a model for studying vacuolar transportation. It is also a suitable cell line for protoplast isolation.
CRPM (S)	MS; with thiamine (0.4 mg/l), no pyridoxine, no nicotinic acid, no glycine, NAA (2 mg/l), kinetin (0.2 mg/l), sucrose (30 g/l), pH 5.8	7	Transfer 20 ml culture into 50 ml medium	Creamy fine cells	
CATL (S)	MS; with NAA (2 mg/l), kinetin (0.2 mg/l, sucrose (30 g/l), pH 5.8	7	Transfer 20 ml culture into 50 ml medium	Yellowish fine cells	• The CATL cell line derived from MP183L cell line, which was originated from A12A2 cell line. • Its parent cell line MP183L was used for protoplast isolation and transformation.

NAA: 1-naphthaleneacetic acid
G10H (G8O): geraniol 10-hydroxylase (also known as geraniol 8-oxidase)
[a]G or S in brackets indicates that the cell lines were grown with glucose or sucrose at 20 or 30 g/l, respectively in the medium.
[b]Except when mentioned otherwise, the medium composition (macro- and micro-nutrients, iron, and vitamins) is according to the MS: Murashige and Skoog (1962); or B5: Gamborg et al. (1968).

Harvesting

Three replicate flasks of each cell line were harvested at the end of their growth cycle (see **Table 1** for growth cycle length), at which the cells were in the stationary phase. The cells were filtered under reduced pressure using a Bűchner funnel. Subsequently, the cells were washed with deionized water, frozen in liquid nitrogen, and lyophilized for 72 hours. The dried cells were kept at room temperature in the dark until further analysis.

Analysis of terpenoid indole alkaloids and precursors

The extraction was adapted from Moreno et al. (1993). Freeze-dried cells (100 mg) from triplicate flasks of each cell lines were extracted two times with 5 ml methanol, vortexed for 10 sec, ultrasonicated for 20 min, and centrifuged at 3,500 rpm for 30 min. The

supernatant was pooled and concentrated to dryness under reduced pressure. The residue was resuspended in 1 ml of 1 M phosphoric acid (H_3PO_4), vortexed for 10 sec, and centrifuged at 13,000 rpm for 10 min. The supernatant was filtered through miracloth before analysis.

Two separate methods for analysis of TIA and TIA precursors were adapted from Tikhomiroff and Jolicoeur (2002). Method **1** was applied for the analysis of TIA (strictosidine, ajmalicine, serpentine, catharanthine, tabersonine, vindoline, vinblastine, and vincristine) using high-performance liquid chromatography (HPLC) on an Agilent Technologies 1200 series chromatograph (Agilent Technologies Inc., Santa Clara, CA, USA). The mobile phase of method **1** consisted of 5 mM Na_2HPO_4 (pH adjusted to 6 with H_3PO_4) (solvent A) and acetonitrile (solvent B). The eluent profile (volume of solvent A/volume of solvent B) was: 0 – 20 min, linear gradient from 80:20 to 20:80; 20 – 25 min, isocratic elution with 20:80 (v/v); 25 – 30 min, linear gradient from 20:80 to 80:20; 30 – 31 min, isocratic elution with 80:20 (v/v). Method **2** was applied for the analysis of TIA precursors (tryptophan, tryptamine, loganic acid, loganin, and secologanin) using a Waters HPLC system (Waters, Milford, MA, USA). The mobile phase of method **2** was 0.01 M H_3PO_4–acetonitrile (85:15, v/v) (isocratic elution). Both methods **1** and **2** were carried out using a Zorbax Eclipse XDB-C18 column (250 mm x 4.6 mm, particle size 5µ) (Agilent Technologies Inc.). The flow rate was 1.5 ml/min and the injection volume was 50 µl for both chromatographic methods. Peak identification was based on a comparison of the retention time and the UV spectrum of the target peaks with those of the standard compounds. Calibration curves of the standard compounds were made for quantitative analysis.

Analysis of carotenoids

Samples were extracted as described by Bino et al. (2005) with some modifications: 70 – 100 mg freeze-dried cells from triplicate flasks of each cell line were extracted with 6 ml of a mixture of methanol, chloroform containing 0.1% butylated hydroxytoluene (BHT) (w/v), and water, in a ratio of 6:5:1 (v/v). The mixture was vortexed for 1 min and left on ice in the dark for 10 min. Subsequently, 3 ml of 50 mM Tris buffer (pH 7.5) containing 1 M sodium chloride and 0.1% BHT (w/v) was added, vortexed for 1 min, and left on ice in the dark for 10 min. Then the mixture was centrifuged (2,500 rpm, 4 ºC, 10 min) and the chloroform phase transferred into a clean, dark vial. The residue was extracted two times with 1.5 ml chloroform (containing 0.1% BHT, w/v) and the chloroform phases were pooled. The extraction was concentrated to dryness under nitrogen gas flow and the residue subsequently redissolved in 1 ml methanol, transferred into an Eppendorf tube, and centrifuged at 13,000

rpm for 5 min. The clear supernatant was collected and analyzed by HPLC adapted from Ben-Amotz et al. (1988).

The chromatography system was carried out using a Vydac 201TP54 C18 column (250 mm x 4.6 mm, particle size 5µ) (Grace, Deerfield, IL, USA) and a Waters HPLC system (Waters). The mobile phase was isocratic elution of 100% methanol (HPLC grade) with a flow-rate of 1 ml/min. The injection volume was 40 µl. Peak identification was performed by comparing the retention time and the UV spectra of reference compounds according to Ben-Amotz et al. (1988) and Taylor et al. (1990). Quantitative analysis was based on the calibration curve of lutein or *β*-carotene.

Analysis of phytosterols

Freeze-dried cells (100 mg) from three replicate flasks of each cell line were weighed, except for the CRPM cell lines (50 mg). One ml of 5*α*-cholestan-3*β*-ol (0.25 mg/ml) was added as an internal standard before saponification of the samples with 5 ml of 1 M potassium hydroxide in ethanol–water (95:5, v/v). Subsequently, the samples were vortexed for 10 sec, ultrasonicated for 5 min, and heated at 80 ^{o}C for 30 min. The samples were cooled to room temperature before centrifuging at 3,500 rpm for 5 min. The solvent was transferred into a clean glass tube with addition of 5 ml water, and extracted two times with 5 ml of diethylether–*n*-hexane (1:1, v/v). The upper layers were pooled, evaporated to dryness, and dissolved in 200 µl *n*-hexane. Subsequently, the solution was cleaned with 10 ml of *n*-hexane followed by 5 ml of *n*-hexane–ethylacetate (95:5, v/v) using a BondElut Silica SPE cartridge (Varian Inc., Lake Forest, CA, USA). The latter fraction was collected and evaporated to dryness. The residue was dissolved in 50 µl *n*-hexane, 200 µl pyridine, and 100 µl *N*,*O*-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane. Subsequently, the mixture was vortexed for 10 sec and heated at 80 ^{o}C for 30 min. The sample was cooled prior to analysis with gas chromatography.

Gas chromatography (GC) equipped with a flame ionization detector (FID) was carried out on an Agilent GC 6890 series (Agilent Technologies Inc.). The column used was a DB-5 (5%-phenyl-methylpolysiloxane) capillary column (30 m length, 0.25 mm internal diameter, film thickness of 0.25 µm) (J&W Scientific Inc., Folsom, CA, USA). The injector temperature was set to 280 ^{o}C, a split ratio of 1:10 and a carrier gas (N_2) flow rate of 1 ml/min. The oven temperature was programmed starting at 200 ^{o}C. The initial temperature began at 200 ^{o}C for 1 min, increased from 200 ^{o}C to 290 ^{o}C at 10 ^{o}C/min and held at 290 ^{o}C for 15 min, providing a total run time of 25 min per sample. The FID detector temperature

was set to 300 ^{o}C. The sample injection volume was 5 µl. Compounds were identified by the retention time compared with those of authentic references. Calibration curves of the standard references were made for quantitative analysis.

Quantitative RT-PCR

Total RNA was isolated from 20 mg of cells and stored at -20^{o}C. DNA contamination was removed using TURBO DNA-free^{TM} kit (Ambion, Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNAs were synthesized from the RNA samples using RevertAid^{TM} (Fisher Scientific, Pittsburgh, PA, USA) oligo (dT) as the primer following the protocol provided by the manufacturer. The cDNAs were used as a template with primers of the specific genes (*HMGR, FPPS, SQS, PDS, DXR, GGPPS, GPS, G10H* or *G8O, TDC, STR, SGD*) listed in **Supplement 1**. The qRT-PCR analysis was performed using a Chromo 4 PTC200 Thermal Cycler (Bio-Rad, Herculas, CA, USA). Ribosomal protein subunit 9 (Rsp9) was used as an internal control and SYBR Green was used in the PCR reactions to quantify the amount of dsDNA. The relative CT (threshold cycle value) method was used to quantify gene expression using MJ Opticon Monitor analysis software version 3.1 (Bio-Rad).

Statistical analysis

Analysis of variance (ANOVA) or *t*-test was performed on SPSS Statistics 17 (SPSS Inc., Chicago, IL, USA) to determine statistical differences ($P < 0.05$). Principal component analysis (PCA) and hierarchical cluster analysis (HCA) were performed on SIMCA-P+ version 12 software (Umetrics, Umeå, Sweden).

Results and Discussion

Analysis of terpenoid indole alkaloids and precursors

The concentrations of terpenoid indole alkaloids (TIA), iridoid and indole precursors in the *C. roseus* cell lines are shown in **Fig. 2**. The indole precursors tryptophan and tryptamine derived from the shikimate pathway were not detected in CRPP (G), CRPP (S) and A11 (S) cell lines, whereas they are found in the other cell lines employed in this study, ranging from 0.14 – 2.97 µmol/g dry weight (DW). Tryptophan (2.97 µmol/g DW) and tryptamine (1.64 µmol/g DW) levels were greater in the CATL (S) compared to the other cell lines ($P < 0.05$). The iridoid precursors, loganin and secologanin were not detected in our cell lines, although the A11 line was previously reported to accumulate secologanin (Contin et al. 1999). However, the precursor of those iridoids, loganic acid was detected in the CRPP

(G) and CRPP (S) cell lines (2.60 – 3.28 µmol/g DW). In addition, both CRPP cell lines accumulated high levels of the alkaloid strictosidine (14.30 – 20.87 µmol/g DW) and serpentine (1.77 – 2.37 µmol/g DW). Compared to the A12A2 (G) cell line, the CRPP (S) produced 85-fold and 156-fold greater strictosidine and serpentine, respectively. This indicates that a high level of iridoid precursor results in depletion of the indole precursors in the CRPP cell lines, whereas in other cell lines, the iridoid pathway was the limiting factor for TIA production, in accordance with previous reports (Moreno et al. 1993; Whitmer et al. 2002).

The antihypertensive compounds; ajmalicine and serpentine are the common mono-TIA found in *C. roseus* cell cultures. Of the cell lines, both CRPP (G) and CRPP (S) accumulated the greatest levels of serpentine (1.77 – 2.37 µmol/g DW), whereas lower levels of serpentine were detected in A11 (G) (0.05 µmol/g DW) and in both A12A2 cell lines (0.02 – 0.57 µmol/g DW). However, ajmalicine was not detected in any of the cell lines employed in this study, even though the accumulation of this compound in the A12A2 and A11 cell lines has been previously reported (Moreno et al. 1993; Contin et al. 1999; El-Sayed and Verpoorte 2002). Serpentine is formed upon vacuolar peroxidase catalyzed oxidation of ajmalicine, for which enzyme activity has been reported to be 20-fold greater in light grown cultures (Blom et al. 1991). As our cell lines were cultivated under continuous light for many years, it is possible that ajmalicine accumulation in these cell lines has been diverted into serpentine.

The highly valuable antineoplastic bisindole alkaloids; anhydrovinblastine, vinblastine, and vincristine were not detected in any of our cell lines. This is due to the lack of vindoline, one of the building blocks of the bisindole alkaloids. Vindoline is not produced in undifferentiated cell cultures of *C. roseus* because its synthesis requires organization of particular cell types found in the aerial tissues (St-Pierre et al. 1999; Murata and De Luca 2005). Biosynthesis of vindoline involves at least six sequential enzymatic reactions starting from the intermediate tabersonine (El-Sayed and Verpoorte 2007). In this study, only CRPP cell lines accumulated tabersonine, as well as another precursor of the bisindole alkaloids, catharanthine. No significant difference was found between the tabersonine levels of CRPP (G) and CRPP (S) cell lines, whereas the level of catharanthine was 1.6-fold higher in the CRPP (S) cell cultures ($P < 0.05$). However, the accumulation ratios between serpentine, tabersonine, and catharanthine seem to be comparable between CRPP (S) and CRPP (G)

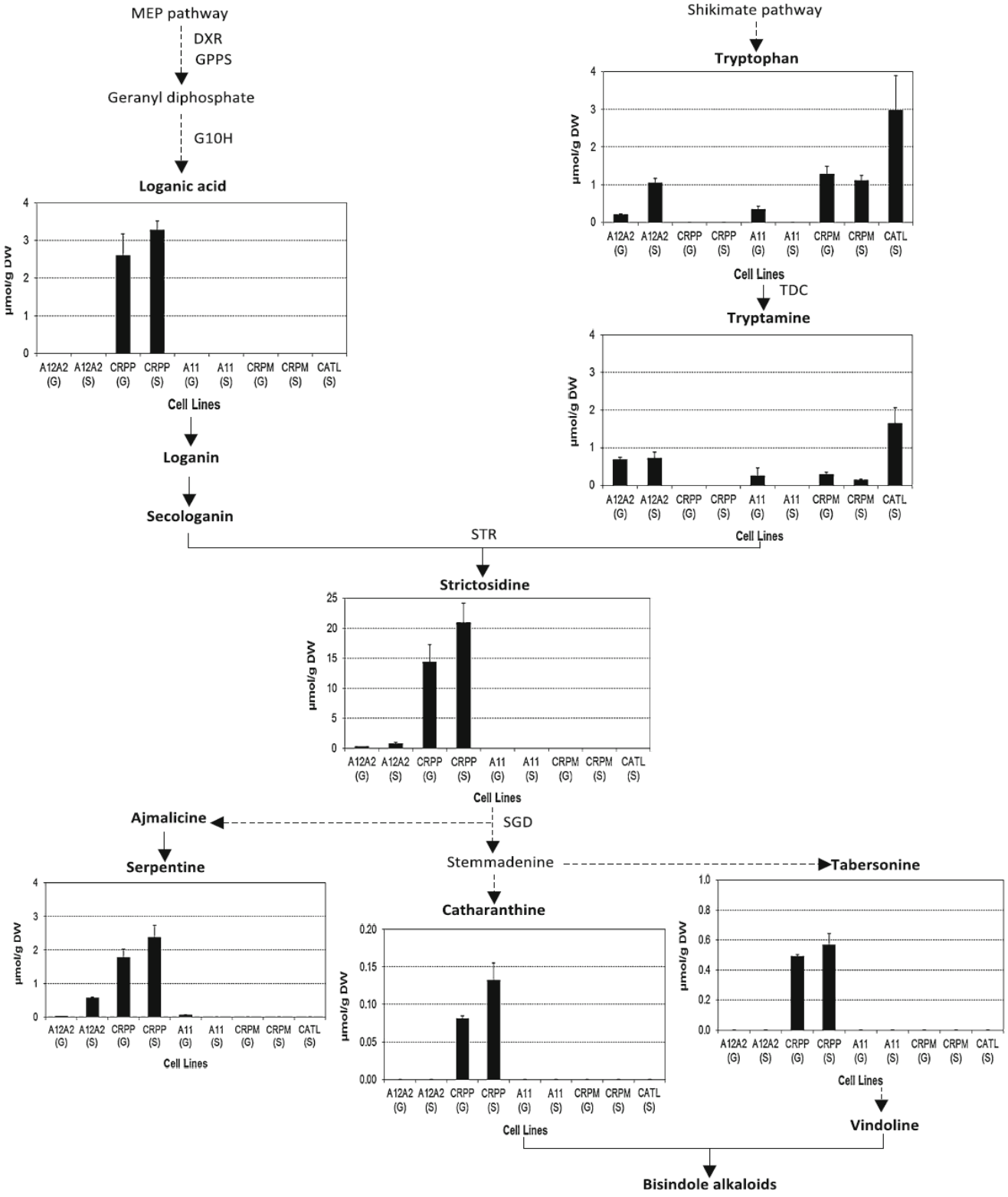

Fig. 2 Analysis of terpenoid indole alkaloids, iridoid and indole precursors in nine *Catharanthus roseus* cell suspension lines. Arrows with dashed lines indicate several biosynthetic steps. DXR: 1-deoxy-D-xylulose-5-phosphate reducto isomerase, GPPS: geranyl diphosphate synthase, G10H: geraniol 10-hydroxylase (G8O: geraniol 8-oxidase), SGD: strictosidine *β*-D-glucosidase, STR: strictosidine synthase, TDC: tryptophan decarboxylase. Error bars represent standard deviation of the mean of three replicates.

lines, i.e. (18:4:1) and (22:6:1), respectively. Apparently, the CRPP cell lines produced greater levels of diverse TIA compared to other cell lines in this study, which makes the CRPP cell lines of a particular interest for bioproduction.

Chung et al. (2011) screened leaves of 64 *C. roseus* cultivars (50 day-old) and showed that 'Cooler Rose Hot' produced the greatest serpentine concentration (1.32 ± 0.13 µmol/g DW). In the present study, the average serpentine concentration of 3 week-old CRPP (G) and CRPP (S) cell lines was 1.77 ± 0.26 µmol/g DW and 2.37 ± 0.36 µmol/g DW, respectively. In addition, the tabersonine levels of the CRPP cell lines (0.49 – 0.57 µmol/g DW) were comparable to that of the 6-week-old Titan Blush cultivar (0.57 µmol/g DW) (Chung et al. 2011). However, the catharanthine level was lower in CRPP cell lines (0.08 – 0.13 µmol/g DW) if compared to the concentrations of the screened *C. roseus* cultivars (0.75 – 8.63 µmol/g DW). The CRPP cell lines accumulated a high level of strictosidine, the first TIA in the biosynthetic pathway. As compared to the 43 *Catharanthus* cultivars analyzed by Hallard (2000), the strictosidine level in the plant leaves was below 5 µmol/g DW, whereas 14.30 µmol/g and 20.87 µmol/g strictosidine were detected in CRPP (G) and CRPP (S) cell lines, respectively.

Analysis of carotenoids

The GGPP pathway towards carotenoid production is a potential competitor for TIA biosynthesis, as both pathways derive from the same precursor (GPP) within the MEP pathway. The accumulation of carotenoids in the *C. roseus* cell suspension lines is shown in **Fig. 3**. Only low levels (< 15 nmol/g DW) of 5,6-epoxy lutein were detected in the CRPM and CATL cell lines, even though its intermediate lutein accumulated at much greater levels in CRPP and A12A2 (S) cell lines ($P < 0.05$). This suggests lutein as the accumulated end-product in this pathway. The earlier precursor of those compounds, i.e. α-carotene, was not detectable in most of the cell lines or detected at trace level (present as tiny peak in the chromatograms) in the CRPP (G) cell line.

In contrast to another pathway, β-carotene was remarkably high in the cell lines, with the trans-β-carotene about 2 – 10 times greater than the *cis*-isomer. Both types of β-carotene as well as the downstream products, violaxanthin and neoxantin, accumulated at greater levels in the A12A2 (S) and both CRPP cell lines ($P < 0.05$) than in the other six cell lines (**Fig. 3**). Zeaxanthin, the intermediate of violaxanthin and neoxanthin, was not detected in

A12A2, CRPP, and A11 cell lines although it was present in low amounts in the CRPM and CATL cell lines. It is presumed that zeaxanthin was fully utilized in A12A2, CRPP, and A11 cell lines to produce violaxanthin and neoxanthin.

The composition of carotenoids in plant leaves is nearly similar in all species: 45% lutein, 25 – 30% *β*-carotene, 15% violaxanthin, 10% neoxanthin, and small amounts of *α*-carotene and zeaxanthin (Namitha and Negi 2010). In our study, *β*-carotene provided the largest part of the composition of carotenoids in *C. roseus* cell suspension cultures, with 88 – 92% in A12A2 and CRPP cell lines, 70 – 78% in CRPM and CATL cell lines, and 48 – 58% in A11 cell lines. Lutein was the second most abundant, with 24 – 26% in the A11 cell lines, 9% in CRPM cell lines, and 5 – 7% in the other cell lines and subsequently, it was followed by neoxanthin and violaxanthin in most of the cell lines.

Analysis of phytosterols

The terpenoid precursor IPP::DMAPP synthesized in the plastidial MEP pathway can be exported to the MVA pathway in the cytosol (Laule et al. 2003). Therefore, the down-stream part of the MVA pathway may also compete with TIA and carotenoid biosynthesis for availability of the central five-carbon (C5) intermediate. In the MVA pathway, plant sterols are synthesized from FPP, which then lead to the production of campesterol via 24-methylenecholesterol or to isofucosterol and subsequently to sitosterol and stigmasterol. Three major phytosterols, i.e. campesterol, stigmasterol, and *β*-sitosterol were analyzed in the *C. roseus* cell lines (**Fig. 4**). In this study, both CRPP cell lines accumulated the greatest levels of campesterol (1.19 – 1.51 µmol/g DW), whereas the lowest level (0.31 µmol/g DW) was in A12A2 (S) ($P < 0.05$). Both A11 cell lines accumulated more *β*-sitosterol than the other cell lines ($P < 0.05$). The lower level of *β*-sitosterol in CRPP (G) seemed to be compensated by a twofold increase in stigmasterol ($P < 0.05$), suggesting an efficient flux towards stigmasterol as accumulated end-product in this cell line. In contrast, little conversion of *β*-sitosterol to stigmasterol was detected in the other cell lines, which resulted in relatively low levels of stigmasterol compared to *β*-sitosterol. In considering the total amount of *β*-sitosterol and stigmasterol versus campesterol, we could deduce that most of the cell lines, i.e. A12A2 (S), CRPP (G), CRPP (S), CRPM (G), and CATL, favored campesterol production, which is in accordance with Suzuki et al. (1995). There were no differences between the levels of campesterol and *β*-sitosterol + stigmasterol in A12A2 (G) and CRPM (S). Although the sum of *β*-sitosterol and stigmasterol levels was greater than campesterol in both A11 cell lines, the results were not statistically significant ($P < 0.05$).

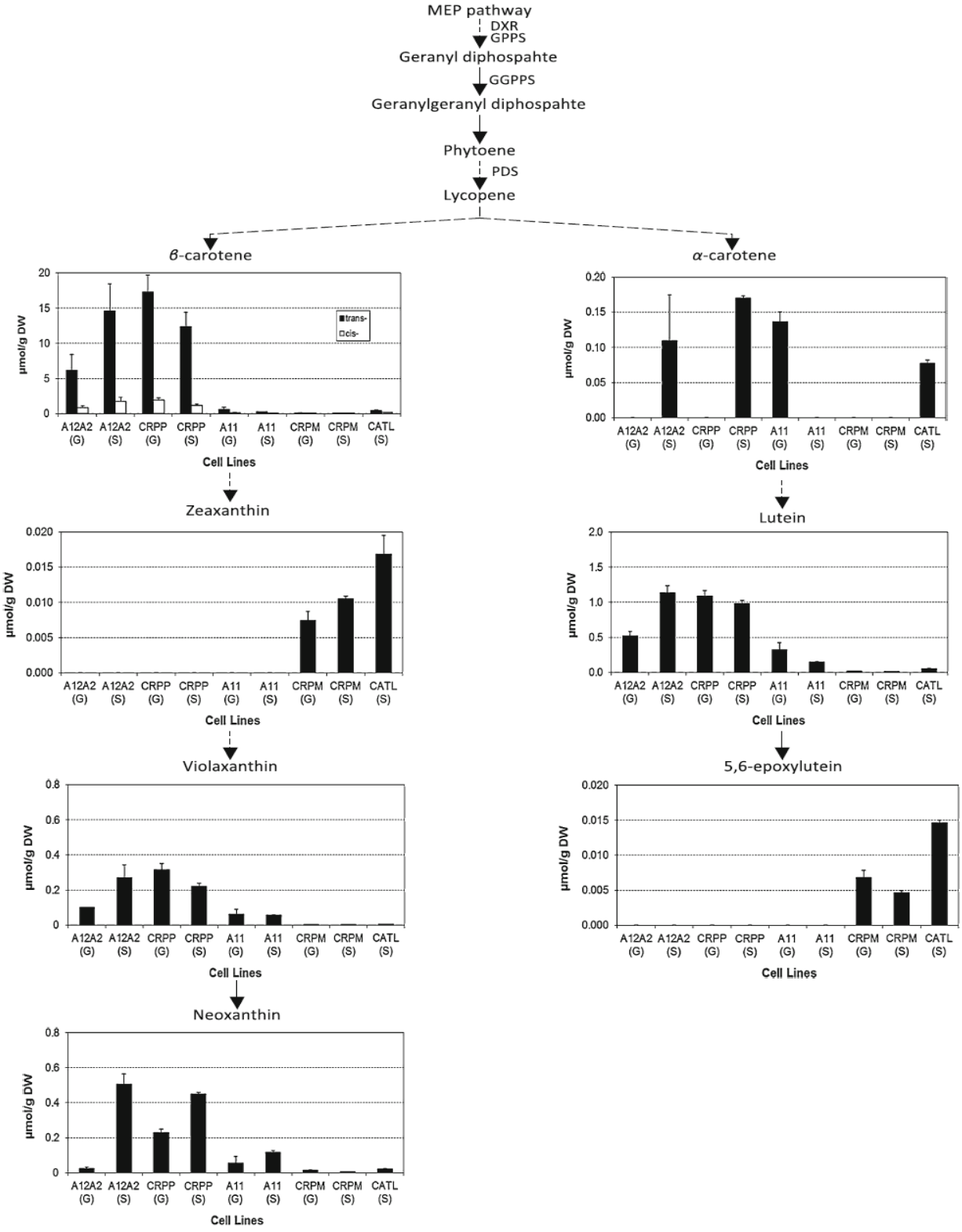

Fig. 3 Carotenoid analysis in nine *Catharanthus roseus* cell suspension lines. Arrows with dashed lines indicate several biosynthetic steps. DXR: 1-deoxy-D-xylulose-5-phosphate reducto isomerase, GPPS: geranyl diphosphate synthase, GGPPS: geranylgeranyl diphosphate synthase, PDS: phytoene desaturase. Error bars represent standard deviation of the mean of three replicates.

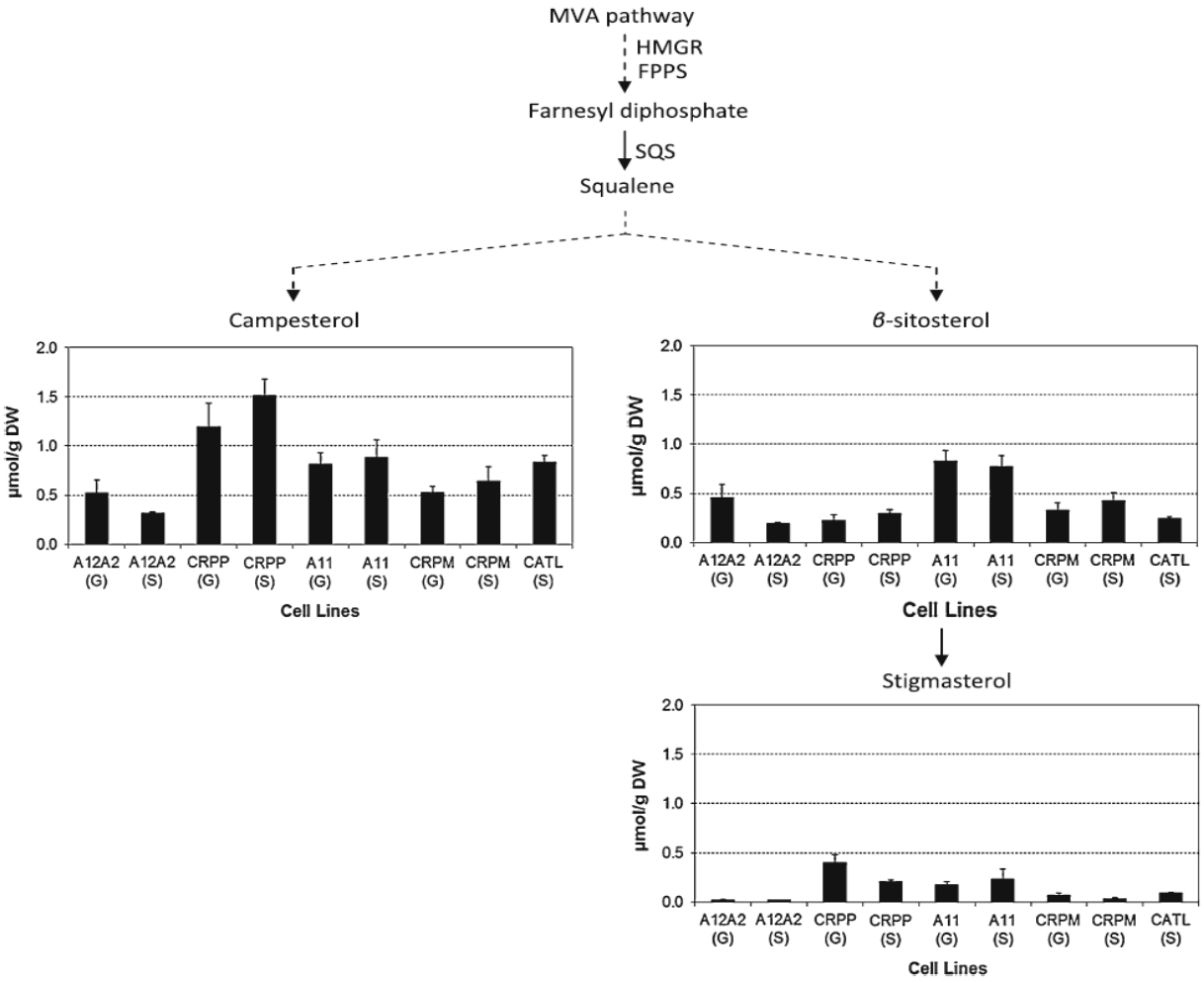

Fig. 4 Sterol analysis in nine *Catharanthus roseus* cell suspension lines. Arrows with dashed lines indicate several biosynthetic steps. HMGR: 3-hydroxy-3-methylglutaryl-CoA reductase, FPPS: farnesyl diphosphate synthase, SQS: squalene synthase. Error bars represent standard deviation of the mean of three replicates.

Total terpenoid accumulation and five-carbon (C5) distribution

Next to analytical evaluation on the individual compounds of TIA, carotenoids, and sterols, the total accumulations of the terpenoid groups were also compared between cell lines (**Fig. 5a**). The TIA level (17 – 24 µmol/g DW) was highly accumulated in CRPP (S) and CRPP (G) cell lines, in which strictosidine provided the largest part of the total TIA composition (86 – 87%). The CRPP cell lines also contained high concentrations of carotenoids (15 – 21 µmol/g DW), whereas the total sterol concentration (1.8 – 2.0 µmol/g DW) was lower than the level of carotenoids and TIA. In A12A2 (G) and A12A2 (S) cell lines, the carotenoid levels were 29- and 14-fold greater, respectively, than those of TIA. In all A11, CRPM, and CATL cell lines, carotenoids were not accumulated more than the levels of sterols. Among the cell lines, CRPP (S) was found to be the most promising TIA-

producing cell line and to have more TIA than carotenoids ($P < 0.05$). Further study needs to be performed to determine whether the terpenoid ratios change at different growth phases. It also remains to be determined whether the TIA:carotenoid ratio can be influenced by external factors, e.g. culture condition and medium composition.

In order to investigate the distribution of five-carbon (C5) precursor in different terpenoid groups from the respective terpenoid biosynthetic pathways, the accumulated levels (in µmol/g DW) of TIA, carotenoids, and sterols were multiplied by the number of C5 units utilized in each group (i.e. TIA = 2 x C5 unit, carotenoid = 8 x C5 unit, and sterol = 6 x C5 unit). The result in **Fig. 5b** shows that the C5 level was greatest in the GGPP pathway towards carotenoid production in all A12A2 and CRPP cell lines. The distribution of C5 was about fourfold (167 µmol/g DW) and twofold (122 µmol/g DW) greater in carotenoids than TIA in CRPP (G) and CRPP (S), respectively. In both CRPP cell lines, the C5 distribution of TIA was significantly greater than sterols ($P < 0.05$). No significant difference of the C5 distribution between TIA and sterols was found in both A12A2 cell lines ($P < 0.05$). Distribution of C5 was three and five times more towards sterols than carotenoids in CRPM (G) and CRPM (S), respectively. A balanced ratio (1:1) of carotenoids:sterols was observed in CATL (S) and A11 (G) cell lines, whereas two times more C5 was distributed into sterols than carotenoids in A11 (S) cell line.

The distribution over the major terpenoid groups measured in this study gives reasonable information of the channeling of C5 into the different terpenoid pathways, even though it is limited in its detail towards other or derived terpenoid groups. Effort had been taken to measure triterpenoids, i.e. *α*-amyrin, *β*-amyrin, and ursolic acid, in the *C. roseus* cell lines. Those compounds were not detected in our cell lines. A group of plant growth regulators, gibberelllins (GAs), are diterpenoids (C20) synthesized in the MEP pathway, which share the same precursor (GPP) with TIA and carotenoids. As a consequence, GAs biosynthesis could be competing with the monoterpenoid (TIA) and tetraterpenoid (carotenoid) pathways. However, GAs were not analyzed in this study. Based on the study performed by Saimoto et al. (1990), the endogenous GA_{12} and GA_{24} detected in the cultured tumor cell of *C. roseus* was 0.08 ng/g FW and 0.4 ng/g FW, respectively. Therefore, if GAs are present at such low levels in our *C. roseus* cell cultures, their biosynthesis is not tapping large amounts of C5 precursor molecules from the total pool if compared with the TIA and carotenoid pathways. However, TIA production might be inhibited at a low level of GAs as Amini et al. (2009) showed an inhibitory effect on ajmalicine accumulation and the down-

regulation of G10H (G8O) gene in the *C. roseus* cell suspension cultures supplied with exogenous gibberellic acid (0.1 – 0.01 µM).

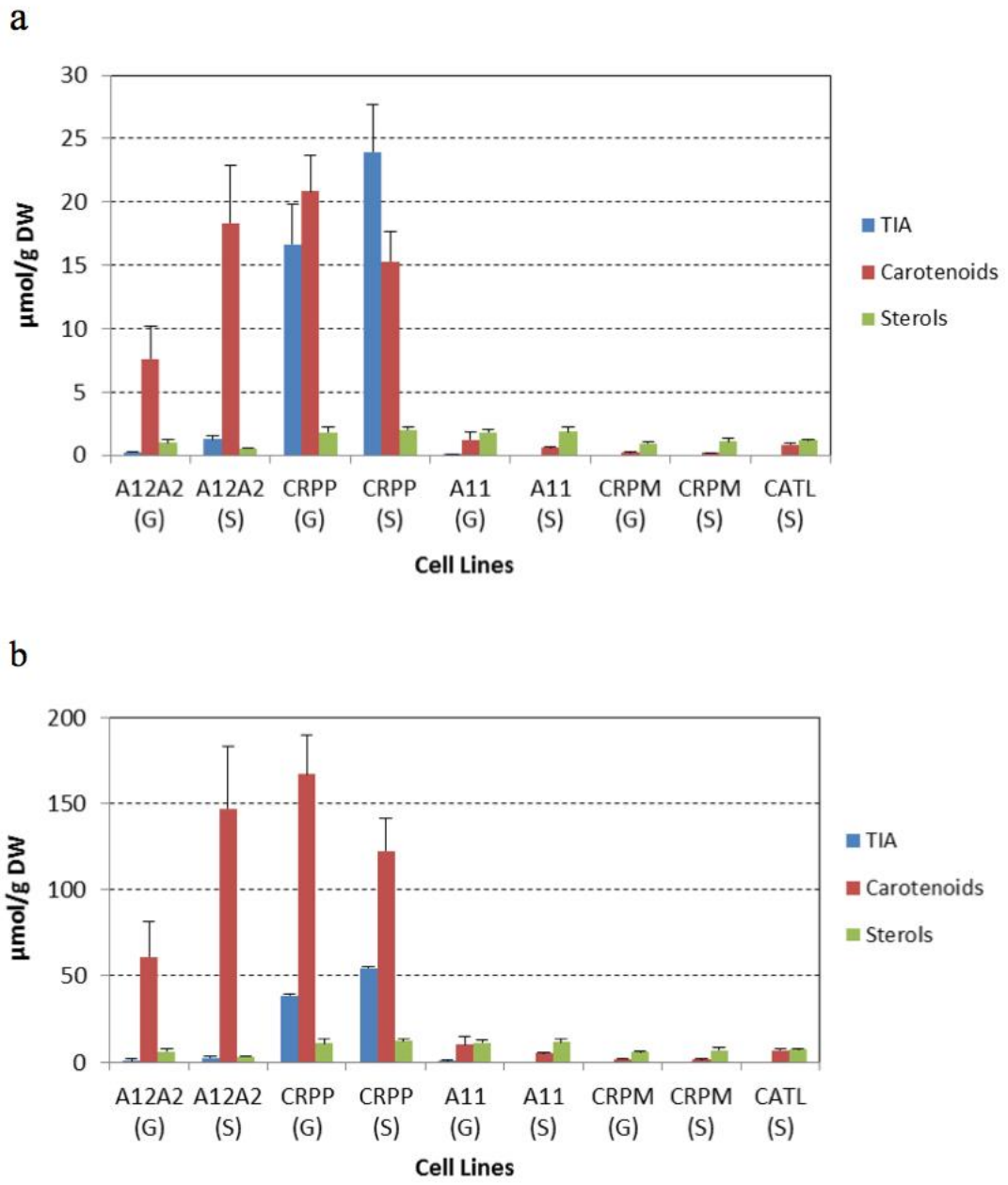

Fig. 5 Total accumulation of terpenoid indole alkaloids, carotenoids, and sterols (a) and distribution of five-carbon (C5) precursor in the respective terpenoid groups (b) in nine *Catharanthus roseus* cell suspension lines. Error bars represent standard deviation of the mean of three replicates.

Cell line characterization using principal component analysis (PCA) and hierarchical cluster analysis (HCA)

In order to distinguish cell lines from one another based on metabolite levels, we applied principal component analysis (PCA) which provides unsupervised clustering method to reduce dimensionality of a multivariate dataset and display systemic variation among samples in an unbiased way. The 18 compounds measured by chromatography are the variables and the observations of the cell lines are based on their µmol/g DW levels. All variables were centered and scaled to unit variance.

The result is presented in a graphical form known as score plot (**Fig. 6a**) and loading plot (**Fig. 6b**). The cell lines were separated by principal component (PC) 1 and 2, which explained 57% and 17% of the variation, respectively. The greatest carotenoid containing cell lines, i.e. CRPP (G), CRPP (S), and A12A2 (S), occurred on the negative side of PC1. The CRPP cell lines accumulating more TIA clustered even more towards the negative side of PC1 than A12A2 (S). The CATL cell line was in the positive quadrant of both PC1 and PC2. According to the loading plot, the CATL could be discriminated from the other cell lines by greater levels of tryptophan, tryptamine, 5,6-epoxy lutein, and zeaxanthin (**Fig. 6b**). Both A11 cell lines were on the positive side of PC1, but on the negative of PC2. These cell lines were characterized by more *β*-sitosterol compared with the other cell lines.

The PCA score plot shows that a cell line grown on a medium with sucrose or glucose cluster closely together, except for the A12A2 cell lines. This suggests that the profiles of observed metabolites of the same type of cell lines are similar and independent of the supplied carbon source. However, the A12A2 (G) and A12A2 (S) cell lines did not group closely which indicates that they differ in their metabolite profiles; we presume that this is mainly due to the difference in the maintenance conditions (see cell line characteristics in **Table 1**). In order to obtain further information on the closeness between groups, we applied hierarchical cluster analysis (HCA) to all component models (9 PCs) from PCA. As shown in **Fig. 6c**, the CRPP-type cell lines which were initiated from a different *C. roseus* cultivar separated into their own group apart from the other cell lines. The CRPM-type cell lines were derived from 58CR cell line, whereas the A12A2 and A11 cell lines derived from the same parental line, i.e. 9CR58, which originated from 58CR cell line. The CATL cell line which clustered close to A12A2 (S) was derived from the A12A2 cell line (**Table 1**). Apparently, the discrimination of the cell lines and the difference in the metabolite profiles could be influenced by both genotype and the cultivation conditions.

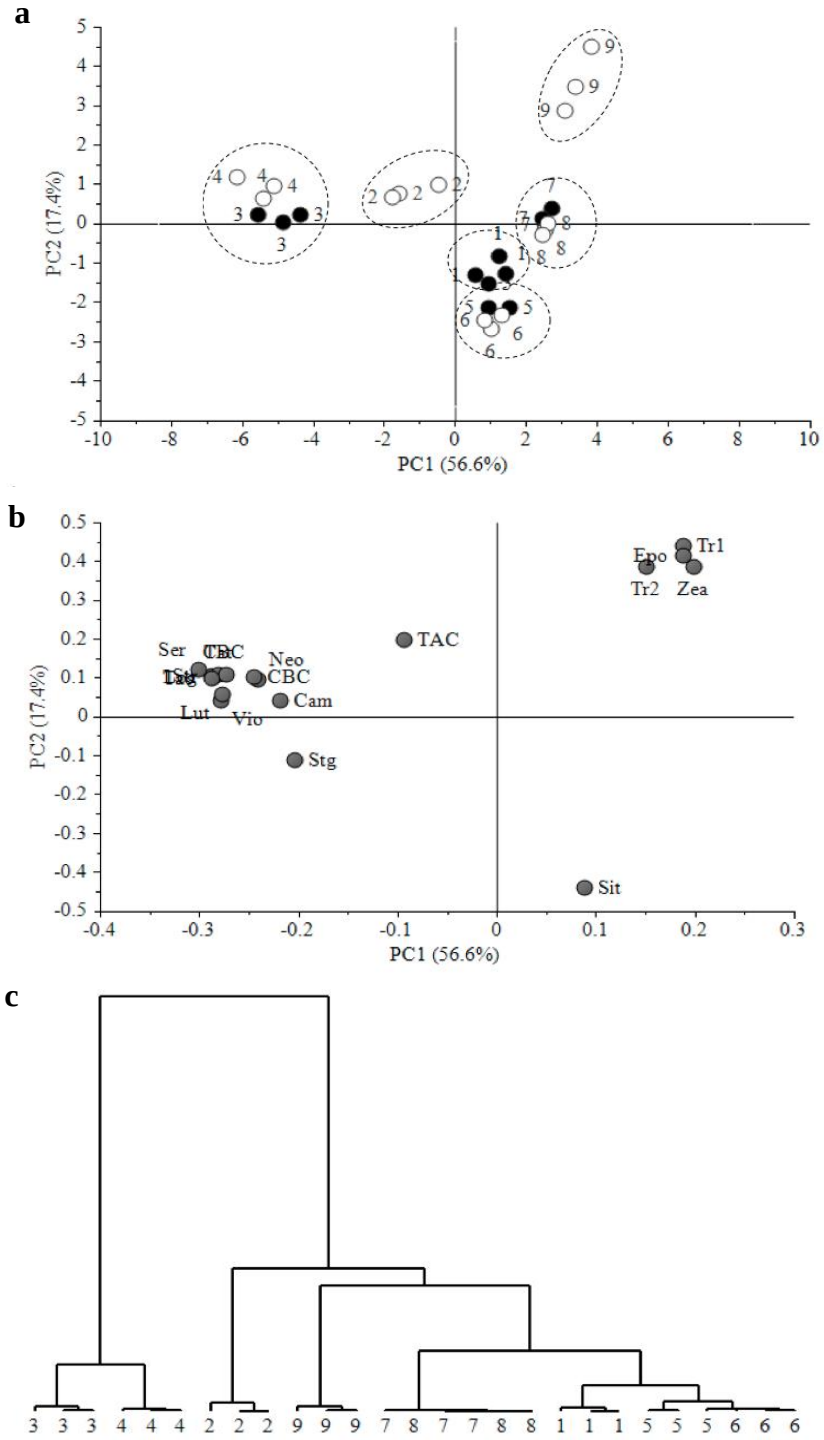

Fig. 6 Principal component analysis (PCA) score (a) and loading (b) plot of nine *Catharanthus roseus* cell suspension lines separated by PC1 and PC2. Dendrogram of hierarchical cluster analysis (HCA) based on nine PCs obtained from PCA (c). 1: A12A2 (G), 2: A12A2 (S), 3: CRPP (G), 4: CRPP (S), 5: A11 (G), 6: A11 (S), 7: CRPM (G), 8: CRPM (S), 9: CATL (S), (○) sucrose, (●) glucose, Tr1: tryptophan, Tr2: tryptamine, Log: loganic acid, Str: strictosidine, Ser: serpentine, Cat: catharanthine, Tab: tabersonine, Cam: campesterol, Stg: stigmasterol, Sit: *β*-sitosterol, Vio: violaxanthin, Neo: neoxanthin, Epo: epoxy-lutein, Lut: lutein, Zea: zeaxanthin, TAC: trans-*α*-carotene, TBC: trans-*β*-carotene, CBC: *cis*-*β*-carotene.

Gene transcript expression

In addition to the metabolite profile, the transcript levels of some genes from the MVA, MEP, and TIA pathways were analyzed from the same samples. The results presented in **Fig. 7** show that both CRPP cell lines expressed geraniol 10-hydroxylase (G10H) gene (also known as geraniol 8-oxidase: G8O) at much greater levels than the other cell lines. The G10H (G8O) enzyme plays a role in catalyzing the conversion of geraniol to 10-hydroxygeraniol (8-hydroxygeraniol), which is an early precursor of the iridoid TIA building block. Collu et al. (2002) showed that G10H (G8O) activity was positively correlated with TIA production. Therefore, it is presumed that the accumulation of loganic acid and TIA in CRPP cell lines was associated with greater transcription levels of G10H (G8O) in the cells. However, a high transcription level does not necessarily represent more enzyme activity, e.g. as availability of cofactors and energy (ATP) are also required. Moreover, the downstream gene transcript levels of loganic acid methyltransferase (LAMT) and secologanin synthase (SLS), which converts loganic acid into loganin and turns loganin into secologanin, respectively, were not measured in this study.

Conversion of tryptophan into tryptamine requires a single enzymatic reaction catalyzed by tryptophan decarboxylase (TDC). The gene transcript level of TDC was highly expressed in the A11 (G) cell line. Whitmer et al. (1998) showed that a high rate of TIA accumulation can occur at low TDC levels and less tryptamine availability which indicate that the rate of TIA biosynthesis depends on rapid turnover of tryptamine rather than on its accumulation, and that high levels of TDC are not required for this rapid turnover to occur. This explains the lack of correlation between TDC activity and TIA biosynthesis. Strictosidine β-D-glucosidase (SGD) is the enzyme converting strictosidine into various TIA. The transcript level of SGD gene was abundant in most of the cell lines, which shows that the cell lines might have high levels of SGD, thus have high capability to produce TIA in the present of substrate.

In the MEP pathway, GPP is converted into GGPP by the enzyme GGPPS. The GGPPS and phytoene desaturase (PDS) are required for carotenoid biosynthesis; the fact that gene transcript levels of both were greater in the CRPP and A12A2 cell lines could link to the high accumulation level of carotenoids in these cell lines. The mRNA levels of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) and squalene synthase (SQS) genes associated with sterol biosynthesis in the MVA pathway were greatest in the A11 (G) cell line.

Although the gene transcript profiles in this study show some association with the metabolite profiles, time-course studies for transcriptome and metabolome are needed to

confirm any hypothesis. Previous studies by Dutta et al. (2005) and Chung et al. (2011) reported contradictionary results. Dutta et al. (2005) showed a positive correlation between transcript levels and TIA levels whereas Chung et al. (2011) did not find such a correlation. Therefore, time-course studies could be helpful to gain a better understanding on the association of transcript levels and metabolites, latter of which are usually lag at least 24 hours behind transcription.

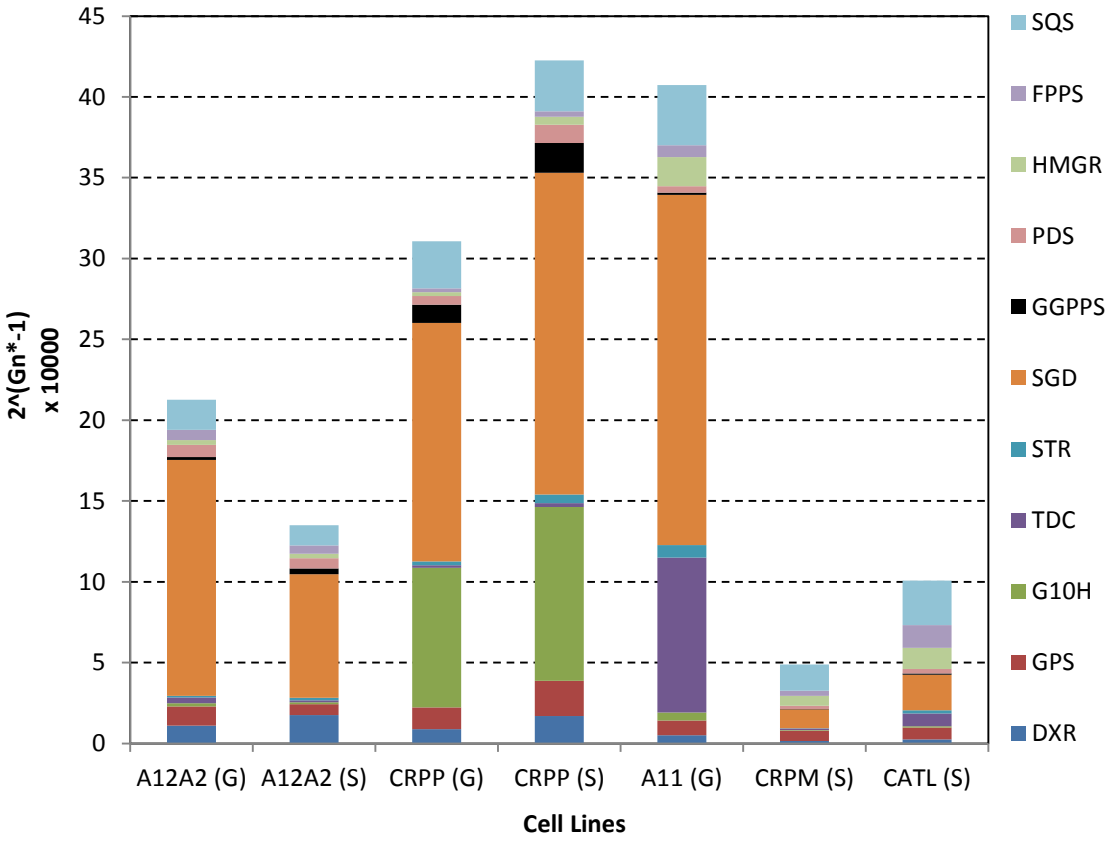

Fig. 7 Transcript levels of some genes from mevalonate (MVA), 2-*C*-methyl-D-erythritol 4-phosphate (MEP), and terpenoid indole alkaloids (TIA) pathways in different *Catharanthus roseus* cell suspension lines. DXR: 1-deoxy-D-xylulose-5-phosphate reducto isomerase, FPPS: farnesyl diphosphate synthase, G10H: geraniol 10-hydroxylase (also known as G8O: geraniol 8-oxidase), GPPS: geranyl diphosphate synthase, GGPPS: geranylgeranyl diphosphate synthase, HMGR: 3-hydroxy-3-methylglutaryl-CoA reductase, PDS: phytoene desaturase, SGD: strictosidine *β*-D-glucosidase, SQS: squalene synthase, STR: strictosidine synthase, TDC: tryptophan decarboxylase.

Conclusion

This study compares the levels of TIA (monoterpenoid) with carotenoids (tetraterpenoid) and sterols (triterpenoid) in several *C. roseus* cell suspension lines. The CRPP (S) cell line showed to be the best TIA-producing cell line and had the best C5

distribution ratio (1:2) between TIA and carotenoids. The production of the latter could be a strong competitor for TIA biosynthesis as both pathways derive from the same precursor (GPP) coming from the MEP pathway. The starting point for monoterpenoid biosynthesis is the geraniol derived from GPP. Increasing the flux from GPP towards geraniol, and thus reduce the production of GGPP, may improve TIA levels in *C. roseus* cell cultures. Sterol production is lower in terms of utilization of the C5 precursors if compared to the TIA and carotenoid in CRPP cell lines. Moreover, even though cross-talk between the pathways may occur at the C5 level, sterols are mainly derived from the mevalonate pathway, and for increasing the flux of precursors to the TIA pathway the sterol pathway seems of less interest.

PCA is useful to chemically distinguish cell lines from each other using quantitative data of metabolite levels. HCA can further refine information on the relationship between cell lines. Higher G10H (or G8O) gene expression levels in the CRPP cell lines seem to be associated with greater TIA accumulation. Nevertheless, time-course studies will be required for a better understanding of this aspect. The metabolite profiles of each cell line are essential not only for information on their chemical composition, but also for better selection of cell lines destined for studies to engineer the terpenoid pathway or to examine interactions of the compounds of interest after a particular treatment of the cell cultures.

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References

Amini A, Glévarec G, Andreu F, Rideau M, Crèche J (2009) Low levels of gibberellic acid control the biosynthesis of ajmalicine in *Catharanthus roseus* cell suspension cultures. Planta Med 75: 187–191

Ben-Amotz A, Lers A, Avron M (1988) Stereoisomers of *β*-carotene and phytoene in the alga *Dunaliella bardawil*. Plant Physiol 86: 1286–1291

Bino RJ, de Vos RCH, Lieberman M, Hall RD, Bovy A, Jonker HH, Tikunov Y, Lommen A, Moco S, Levin I (2005) The light-hyperresponsive *high pigment-2^{dg}* mutation of tomato: alterations in the fruit metabolome. New Phytol 166: 427–438

Blom TJM, Sierra M, van Vliet TB, Franke-van Dijk MEI, de Koning P, van Iren F, Verpoorte R, Libbenga KR (1991) Uptake and accumulation of ajmalicine into isolated vacuoles of cultured cells of *Catharanthus roseus* (L.) G. Don. and its conversion into serpentine. Planta 183: 170–177

Chung IM, Kim EH, Li M, Peebles CAM, Jung WS, Song HK, Ahn JK, San KY (2011) Screening 64 cultivars *Catharanthus roseus* for the production of vindoline, catharanthine, and serpentine. Biotechnol Prog 27: 937–943

Collu G, Garcia AA, van der Heijden R, Verpoorte R (2002) Activity of the cytochrome P450 enzyme geraniol 10-hydroxylase and alkaloid production in plant cell cultures. Plant Sci 162: 165–172

Contin A, Collu G, van der Heijden R, Verpoorte R (1999) The effects of phenobarbital and ketoconazole on the alkaloid biosynthesis in *Catharanthus roseus* cell suspension cultures. Plant Physiol Biochem 37: 139–144

Courdavault V, Burlat V, St-Pierre B, Giglioli-Guivarc'h N (2005) Characterisation of CaaX-prenyltransferases in *Catharanthus roseus*: relationships with the expression of genes involved in the early stages of monoterpenoid biosynthetic pathway. Plant Sci 168: 1097–1107

Dutta A, Batra J, Pandey-Rai S, Singh D, Kuar S, Sen J (2005) Expression of terpenoid indole alkaloid biosynthethic pathway genes corresponds to accumulation of related alkaloids in *Catharanthus roseus* (L.) G. Don. Planta 220: 376–383

El-Sayed M, Verpoorte R (2002) Effect of phytohormones on growth and alkaloid accumulation by a *Catharanthus roseus* cell suspension cultures fed with alkaloid precursors tryptamine and loganin. Plant Cell Tiss Org Cult 68: 265–270

El-Sayed M, Verpoorte R (2007) *Catharanthus* terpenoid indole alkaloids: biosynthesis and regulation. Phytochem Rev 6: 277–305

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50: 155–158

Glenn WS, Runguphan W, O'Connor SE (2013) Recent progress in the metabolic engineering of alkaloids in plant systems. Curr Opin Biotechnol 24: 354–365

Hallard DAC (2000) Transgenic plant cells for the production of indole alkaloids. PhD Thesis, Leiden University, The Netherlands. Chapter 7, Extraction and liquid chromatography photodiode array detection tandem mass spectrometry analysis of *Catharanthus* alkaloids, pp. 88–109. ISBN 9074538495

Hemmerlin A, Hoeffler JF, Meyer O, Tritsch D, Kagan IA, Grosdemange-Billiard C, Rohmer M, Bach TJ (2003) Cross-talk between the cytosolic mevalonate and the plastidial methylerythritol phosphate pathways in tobacco bright yellow-2 cells. J Biol Chem 278: 26666–26676

Laule O, Fürholz A, Chang H-S, Zhu T, Wang X, Heifetz PB, Gruissem W, Lange BM (2003) Crosstalk between cytosolic and plastidial pathways of isoprenoid biosynthesis in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 100: 6866–6871

Moreno PRH, van der Heijden R, Verpoorte R (1993) Effect of terpenoid precursor feeding and elicitation on formation of indole alkaloids in cell suspension cultures of *Catharanthus roseus*. Plant Cell Rep 12: 702–705

Moreno PRH, van der Heijden R, Verpoorte R (1995) Cell and tissue cultures of *Catharanthus roseus*: a literature survey II. Updating from 1988 to 1993. Plant Cell Tiss Organ Cult 42:1–25

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473–497

Murata J, De Luca V (2005) Localization of tabersonine 16-hydroxylase and 16-OH tabersonine-16-*O*-methyltransferase to leaf epidermal cells defines them as a major site of precursor biosynthesis in the vindoline pathway in *Catharanthus roseus*. Plant J 44: 581–594

Mustafa NR, Kim Hye Kyong, Choi YH, Verpoorte R (2009) Metabolic changes of salicylic acid-elicited *Catharanthus roseus* cell suspension cultures monitored by NMR-based metabolomics. Biotechnol Lett 31: 1967–1974

Mustafa NR, Verpoorte (2007) Phenolic compounds in *Catharanthus roseus*. Phytochem Rev 6: 243–258

Mustafa NR, de Winter W, van Iren F, Verpoorte R (2011) Initiation, growth and cryopreservation of plant cell suspension cultures. Nat Protoc 6: 715-742

Namitha KK, Negi PS (2010) Chemistry and biotechnology of carotenoids. Crit Rev Food Sci Nutr 50: 728–760

O'Connor SE (2012) Strategies for engineering plant natural products: the iridoid-derived monoterpene indole alkaloids of *Catharanthus roseus*. Methods Enzymol 515: 189–206

Pati PK, Kaur J, Singh P (2011) A liquid culture system for shoot proliferation and analysis of pharmaceutically active constituents of *Catharanthus roseus* (L.) G. Don. Plant Cell Tiss Organ Cult 105: 299–307

Pietrosiuk A, Furmanowa M, Łata B (2007) *Catharanthus roseus*: micropropagation and in vitro techniques. Phytochem Rev 6: 459–473

Rohmer M (1999) The discovery of a mevalonate-independent pathway for isoprenoid biosynthesis in bacteria, algae and higher plants. Nat Prod Rep 16: 565–574

Salim V, De Luca V (2013) Towards complete elucidation of monoterpene indole alkaloid biosynthesis pathway: *Catharanthus roseus* as a pioneer system. In: Giglioli-Guivarc'h N (ed.) Advances of Botanical Research – New Light on Alkaloid Biosynthesis and Future Prospects, Academic Press, London, pp. 1–37

Saimoto H, Nakagawa S, Kobayashi M, Fujioka S, Barreto MCC, Sakurai A, Syono K (1990) Endogenous levels of gibberellins, IAA and cytokinins in *Catharanthus* crown gall tissues of different tumor types. Plant Cell Physiol 31: 365–370

Schuhr CA , Radykewicz1 T, Sagner S, Latzel C, Zenk MH, Arigoni D, Bacher A, Rohdich F, Eisenreich W (2003) Quantitative assessment of crosstalk between the two isoprenoid biosynthesis pathways in plants by NMR spectroscopy. Phytochem Rev 2: 3–16

Suzuki H, Inoue T, Fujioka S, Saito T, Takatsuto S, Yokota T, Murofushi N, Yanagisawa T, Sakurai A (1995) Conversion of 24-methylcholesterol to 6-oxo-24-methylcholestanol, a putative intermediate of the biosynthesis of brassinosteroids, in cultured cells of *Cathanranthus roseus.* Phytochemistry 40: 1391–1397

St-Pierre B, Vazquez-Flota F, De Luca V (1999) Multicellular compartmentation of *Catharanthus roseus* alkaloid biosynthesis predicts intercellular translocation of pathway intermediate. Plant Cell 11: 887–900

Taylor RF, Farrow PE, Yelle LM, Harris JC, Marenchic IG (1990) Advances in HPLC and HPLC-MS of carotenoids and retinoids. In: Krinsky NI, Mathews-Roth MM, Taylor RF (eds.) Carotenoids: Chemistry and Biology, Plenum Press, New York, pp. 105–123

Tikhomiroff C, Jolicoeur M (2002) Screening of *Catharanthus roseus* secondary metabolites by high-performance liquid chromatography. J Chromatogr A 955: 87–93

van der Heijden R, Jacobs DI, Snoeijer W, Hallard D, Verpoorte R (2004) The *Catharanthus* alkaloids: pharmacognosy and biotechnology. Curr Med Chem 11: 607– 628

Verpoorte R, Contin A, Memelink J (2002) Biotechnology for the production of plant secondary metabolites. Phytochem Rev 1: 13–25

Whitmer S, Canel C, Hallard D, Goncalves C, Verpoorte R (1998) Influence of precursor availability on alkaloid accumulation by transgenic cell line of *Catharanthus roseus*. Plant Physiol 116: 853–857

Whitmer S, van der Heijden R, Verpoorte R (2002) Effect of precursor feeding on alkaloid accumulation by a strictosidine synthase over-expressing transgenic cell line S1 of *Catharanthus roseus*. Plant Cell Tiss Organ Cult 69: 85–93

Zhao J, Verpoorte R (2007) Manipulating indole alkaloid production by *Catharanthus roseus* cell cultures in bioreactors: from biochemical processing to metabolic engineering. Phytochem Rev 6: 435– 457

Zhou ML, Shao JR, Tang YX (2009) Production and metabolic engineering of terpenoid indole alkaloids in cell cultures of the medicinal plant *Catharanthus roseus* (L.) G. Don (Madagascar periwinkle). Biotechnol Appl Biochem 52: 313–323

Supplement 1 List of primers for quantitative RT-PCR

Gene	Product size (bp)	Primer sequences	
	cDNA	Forward	Reverse
HMGR	162	5′-GAGATGGAATGACTAGGGCTCC-3′	5′-GCAATTGCACATTGGACGCT-3′
DXR	147	5′-TTGGCCCCGCCTTGATCTTT-3′	5′-GCCTTCTCATTTGCTGCACT-3′
GPS	299	5′-TGAAACAAGGCGAGGGATTG-3′	5′-GATGTTTGCCCAGCAAGAAG-3′
PDS	309	5′-GCAGTTGGACTCTTGCCG-3′	5′-GCACTTCACCTCCTAGTGATCG-3′
GGPPS	264	5′-ATGCGCTCTAATCTTTGTCACCC-3′	5′-CCCTAACCAAGACTGCATCTTCC-3′
G10H (G8O)	247	5′-GGTAGCCTCACGATGGAGAA-3′	5′-CCTTGGCAGAATCCGAATAA-3′
TDC	227	5′-CGCCTGTATATGTCCCGAGT-3′	5′-GTTGCGATTTGCCAATTTTT-3′
SGD	246	5′-ATTTGCACCAGGAAGAGGTG-3′	5′-TATGAACCATCCGAGCATGA-3′
FPPS	160	5′-GGCTGGTTGTGAAAGCATTAG-3′	5′-CTCAAACACACCCTGAAGATCAAG-3′
SQS	265	5′-TATTTGGTTCTTCGAGCTCTTGACAC-3′	5′-ATATTTCGATTCTGTGCTATTCCACA-3′
STR	146	5′-TGCCACACAACTAGCCACAA-3′	5′-TCATGATTTCTTCCACACCTTCG-3′
Rsp9	190	5'-TCCACCATGCCAGAGTGCTCATTAGG-3′	5'-TCCATCACCACCAGATGCCTTCTTCG-3'

Chapter 3

Metabolic alterations and distribution of five-carbon terpenoid precursors in jasmonic acid-elicited *Catharanthus roseus* cell suspension cultures

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Abstract

Catharanthus roseus produces an important group of secondary metabolites known as terpenoid indole alkaloid (TIA). In *C. roseus* cell cultures, the pathway of the terpenoid moiety is considered as the limiting factor in TIA production. The pathway of terpenoidal precursors in TIA is strongly linked with other terpenoid pathways, which suggests that TIA production might be limited by competition for the isopentenyl precursors. This raises the question whether the stimulation of TIA production by certain signal molecules is due to a redistribution of precursors between the associated terpenoid pathways and/or to a total increase of precursor availability. To investigate the effect of a TIA-increasing signal molecule, the cell suspension cultures of *C. roseus* were elicited with jasmonic acid (JA) and the metabolic changes of different terpenoid pathways were evaluated targeting on TIA (monoterpenoid; C10), carotenoid (tetraterpenoid; C40), and phytosterol (triterpenoid; C30). The results of HPLC-DAD or GC-FID analyses showed that TIA and carotenoid levels almost doubled upon JA elicitation, while phytosterol levels remained constant if compared to the control. This indicates that only these two pathways benefit from an increased flow in the MEP pathway, and that export of IPP::DMAPP intermediates to the cytosolic terpenoid routes like the phytosterol is minimal. Furthermore, the metabolites profiles observed by ^{1}H-NMR spectroscopy and analyzed by multivariate data analysis showed a discrimination of JA-elicited and control cells between 24 – 72 hours after treatments. In JA-elicited cells, the levels of strictosidine and organic acids in the TCA cycle, i.e. succinic acid, fumaric acid, and malic acid were increased, whereas sugars levels were decreased. This study portrays metabolic alterations upon JA elicitation in different biosynthetic pathways, which provides a knowledge platform for developing strategies to engineer fluxes in a complex biosynthetic network in order to obtain high TIA-producing *C. roseus* cell lines.

Introduction

Catharanthus roseus plants produce a diverse array of secondary metabolites including terpenoid indole alkaloids (TIA), which are a valuable and unique source of the pharmaceutically important anticancer compounds vincristine and vinblastine. The other important TIA produced by *C. roseus* are ajmalicine and serpentine having antihypertensive activity (Moreno et al. 1995; van der Heijden et al. 2004).

Plants require a long cultivation period and produce only low amounts of secondary metabolites. Therefore, cell culture systems are explored as an alternative source of valuable plant metabolites. Despite some successful examples (Verpoorte et al. 2002), the production

of secondary metabolites in most plant cell cultures is relatively low or sometimes zero. In the case of *C. roseus*, bisindole alkaloids such as vincristine and vinblastine are not produced in cell cultures due to the lack of one of the biosynthetic precursor, vindoline. Nevertheless, semi-synthesis of bisindole alkaloids is possible by coupling vindoline and catharanthine, thus optimizing cell cultures to produce the individual precursors is an interesting target. Although ajmalicine and serpentine accumulate in *C. roseus* cell cultures, the productivity is too low compared to the intact plants for a cost-competitive mass production (Zhao and Verpoorte 2007).

Several strategies can be applied to improve product yields in plant cell cultures. One of the techniques is to induce the biosynthesis of secondary metabolites by elicitation. Elicitation strategies using exogenous jasmonic acid (JA) or its volatile methyl ester, methyl jasmonic (MeJA), often result in elevated levels of certain secondary metabolites. In the elicitation process, jasmonates play an important role as regulatory signals to induce *de novo* transcription and translation leading to the induction of secondary metabolite biosynthesis in plant cell cultures (Gundlach et al. 1992; Memelink et al. 2001; Zhao and Verpoorte 2007). Exogenous application of jasmonates to *C. roseus* cell cultures (El-Sayed and Verpoorte 2002; Lee-Parsons and Royce 2006; Vázquez-Flota et al. 2009), hairy roots (Rijhwani and Shanks 1998; Vázquez-Flota et al. 2009), shoot cultures (Vázquez-Flota et al. 2009) and seedlings (El-Sayed and Verpoorte 2004) increased the production of TIA caused by an elevated expression of a set of biosynthesis related genes (Memelink et al. 2001). Combination of transcript and 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). Therefore, JA treatment is a useful biochemical tool to stimulate the production and to study the regulation of TIA biosynthesis.

Terpenoid indole alkaloids are derived from strictosidine which is formed by the coupling of the indole tryptamine and the iridoid secologanin, in which the latter are considered as the limiting factor of TIA biosynthesis in *C. roseus* cell cultures (Moreno et al. 1993; Whitmer et al. 2002). One of the limitations could be the competition at the level of IPP::DMAPP, the precursors of different terpenoid groups such as monoterpenoids (C10), triterpenoids (C30) or tetraterpenoids (C40). Furthermore, several studies showed an interaction between the precursor pools in cytosolic mevalonate and plastidial MEP pathway (Schuhr et al. 2003; Hemmerlin et al. 2003; Laule et al. 2003).

In this study, the effect of jasmonic acid elicitation on different terpenoid pathways, i.e. monoterpenoids (TIA), triterpenoids (phytosterols), and tetraterpenoids (carotenoids), and

the distribution of five-carbon precursors (C5) into representative terpenoid groups were evaluated. The transient accumulation of TIA, carotenoid, and phytosterol were analyzed by high performance liquid chromatography-diode array detector (HPLC-DAD) or gas chromatography-flame ionization detector (GC-FID). In addition, the metabolomic changes between control and treated samples were analyzed with our NMR-based metabolomics platform.

Materials and Methods

Cell culture materials

The *Catharanthus roseus* cell suspension culture (CRPP cell line) was obtained from the Natural Products Laboratory, Institute of Biology, Leiden University, The Netherlands. The cell suspension culture was subcultured every three weeks by diluting the cultures with fresh medium (1:1) consisting of B5 medium (Gamborg et al. 1968) supplemented with 1.86 mg/l NAA and 20 g/l glucose. The medium was adjusted to pH 5.8 before autoclaving at 121 ^{o}C for 20 min. The cultures were maintained in 250 ml shake flasks closed with T-32 silicon foam stoppers (Shin Etsu, Tokyo, Japan) and placed on a gyratory shaker (110 rpm) at 25 ^{o}C in continuous light (10.8 – 27 µE/m^2/sec). For the experiment, 20 ml (approx. 4 g fresh weight) of a two-week-old cell suspension culture was inoculated into 50 ml culture medium in the same cultivation conditions for 5 days prior to elicitation.

Elicitation

The stock solution of jasmonic acid (10 mg/ml) was prepared by dissolving jasmonic acid (Sigma-Aldrich Chemie, Steinheim, Germany) in 2 ml ethanol and diluted with sterile water to acquire the total volume of 10 ml. The solution was mixed and filter sterilized through 0.22 µm Millex^{TM} filter (Millipore, Bedford, MA, USA). At the fifth day of culture, 50 µl of jasmonic acid solution was added into the cell culture (70 ml) to achieve the final concentration of 34 µM (0.5 mg/flask). The same amount of control solution (20% v/v ethanol) was applied to the cell cultures. The treated and control cultures were performed in triplicate flasks and harvested at 0, 2, 6, 24, 48, and 72 hours after elicitation. The cells were filtered under reduced pressure, washed three times with deionized water, and lyophilized for 72 hours.

Analysis of terpenoid indole alkaloids, carotenoids, and sterols

Analysis of terpenoid indole alkaloids, carotenoids, and sterols were carried out according to the methods described by Saiman et al. (2014) (Chapter 2). Briefly, 100 mg of the freeze-dried cells were weighed and extracted for analyzing TIA and sterols, while 70 mg freeze-dried cells were extracted for carotenoids. Analyses of carotenoids, TIA and TIA precursors were performed using different HPLC-DAD methods, whereas the GC-FID system was used to analyze sterols. Calibration curves of the standard compounds were made for quantitative analyses. Results were presented as an average of the analyses of two separate flasks for several time-points (0, 6, 24, and 72 hours after treatment).

NMR measurement

NMR analysis was conducted according to the protocol of Kim et al. (2010). The freeze-dried cells (25 mg) from triplicate flasks of each treatments (JA and control) and time-points (0, 2, 6, 24, 48, 72 hours) were extracted with 1.2 ml of CD_3OD and 0.3 ml of KH_2PO_4 buffer in D_2O (pH 6.0, containing 0.01% w/w trimethylsilyl propanoic acid (TMSP) as internal standard). The mixture was vortexed for 10 sec, sonicated for 10 min, and centrifuged for 15 min (14,000 rpm). The supernatant was transferred into NMR tube for measurement of ^{1}H-NMR using a Bruker AV 600 MHz spectrometer (Bruker, Karlsruhe, Germany) with cryoprobe. The ^{1}H-NMR spectra were recorded at 25 ^{o}C, consisted of 128 scans requiring 10 min and 26 sec acquisition time with following parameters: 0.16 Hz/point, pulse width of 30 (11.3 µs), and relaxation delay of 1.5 sec. Methanol-d_4 was used as the internal lock. A presaturation sequence was used to suppress the residual water signal with low power selective irradiation at the water frequency during the recycle delay. Free induction decay was Fourier transformed with a line-broadening (LB) factor of 0.3 Hz.

The resulting spectra were manually phased, baseline corrected, and calibrated to TMSP at 0.0 ppm by using XWIN NMR version 3.5 (Bruker). The AMIX software (Bruker) was used to reduce the ^{1}H-NMR spectra to an ASCII file, with total intensity scaling. Bucketing or binning was performed and the spectral data were reduced to include regions of equal width (δ 0.04) corresponding to the region of δ 0.40 – 10.00. The regions of δ 4.75 – 4.90 and δ 3.30 – 3.35 were not included in the analysis because of the remaining signal of D_2O and CD_3OD, respectively. Multivariate data analysis was performed with the SIMCA-P+ software version 12.0 (Umetrics, Umeå, Sweden). The *t*-test was performed using IBM SPSS Statistics 20 (SPSS Inc., Chicago, IL, USA) to determine statistical significance ($P < 0.05$) of the relative levels of metabolites.

Results and Discussion

Cell culture material

In the previous chapter, several *C. roseus* cell lines were analyzed for the accumulation of different terpenoid groups. The CRPP cell lines were found to have high productivity in terms of quantity and diversity of TIA and terpenoid compounds as compared to the other cell lines. Therefore, we used the CRPP cell line as a model to study the effect of jasmonic acid (JA) elicitation towards the accumulation of different terpenoid end-products derived from the mevalonate and MEP pathways including the distribution of five-carbon precursors (C5). Even though the sucrose supplemented CRPP cell line had a slightly higher TIA content, the glucose line was preferred for this study as we may pursue feeding with labeled glucose in later experiments to determine the fluxes of the carbons through the mevalonate route and the MEP pathway.

To estimate the best time-point for elicitation studies, the growth curve of the CRPP cell line was determined (**Fig 1**). Based on the cell growth curve, the elicitation experiment was performed at day 5, as the maximum biomass is reached and high metabolic rates can be achieved.

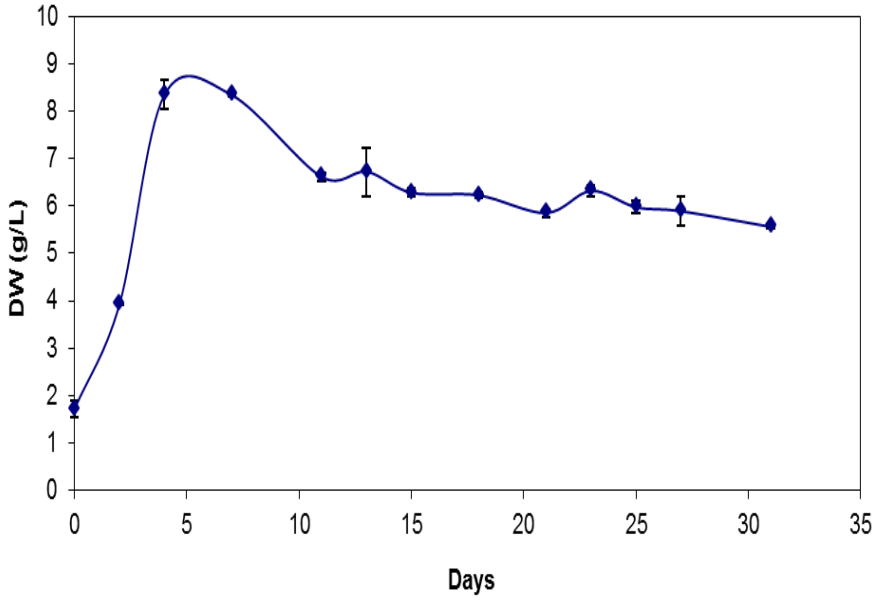


Fig. 1 The biomass accumulation of CRPP (glucose) cell line at different time-points after inoculating 20 ml of a three-week-old culture into 50 ml fresh medium. The cell dry weight (DW) was determined from duplicate flasks; error bars indicate the two values.

Analysis of terpenoid indole alkaloids and precursors

The analysis of iridoids showed only the presence of loganic acid, whereas loganin and secologanin, if present, were below detection limit. Neither tryptophan nor tryptamine was detected in the cell suspension culture. TIA analysis only showed strictosidine as major alkaloid and smaller amounts of serpentine, ajmalicine, and tabersonine. **Figure 2** shows the levels of loganic acid and TIAs at different time-points after jasmonic acid elicitation. Loganic acid, the iridoid precursor of TIA was twofold higher in the JA-elicited cells 6 hours after elicitation. Although loganic acid concentration was still higher compared to the control

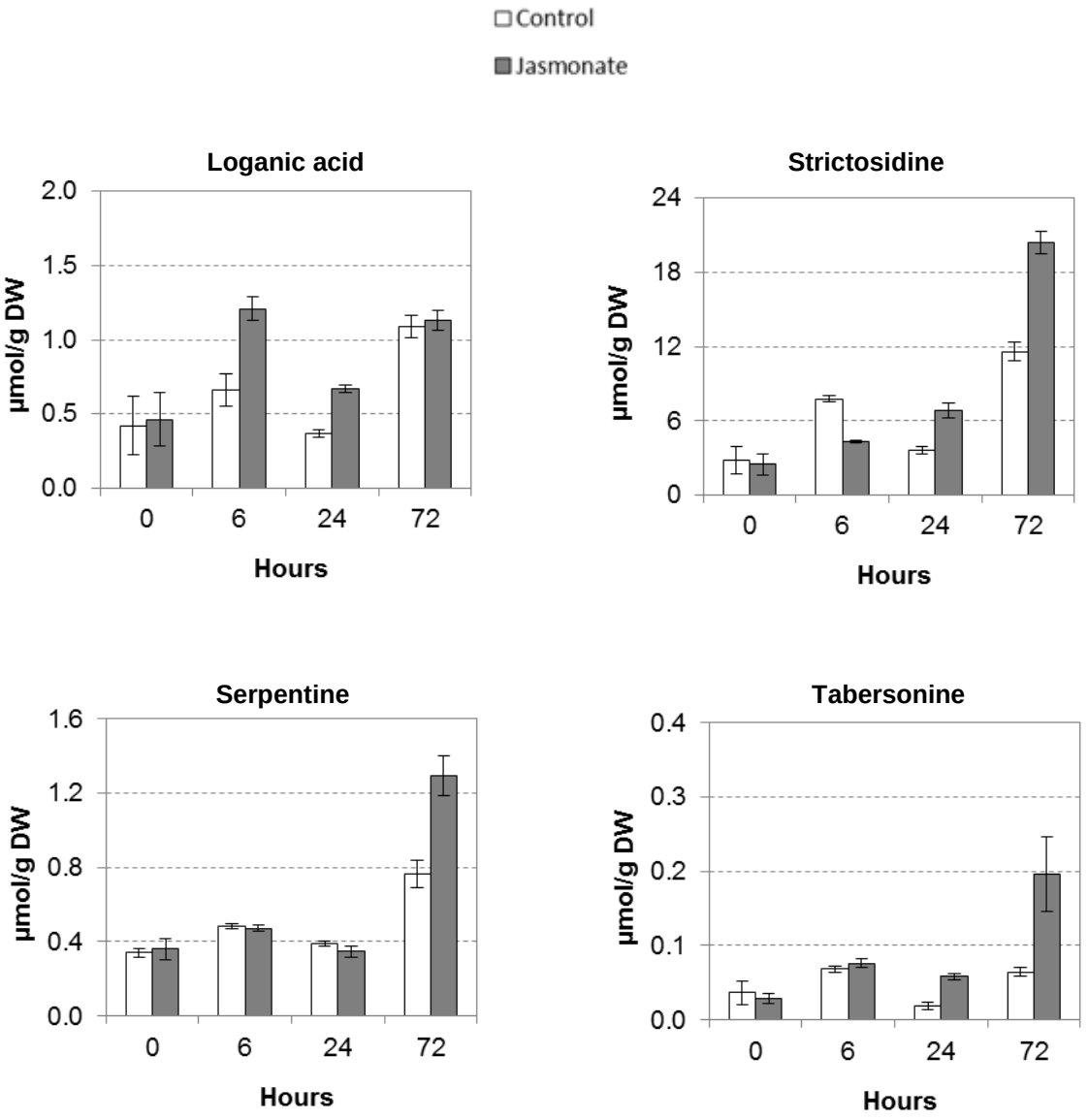


Fig. 2 Time course of loganic acid and terpenoid indole alkaloid production in CRPP cell line elicited with 34 µM jasmonic acid or control solution measured by HPLC-Diode Array Detector (DAD). Results are the mean of two replicates; error bars indicate the two values.

at 24 hours, the level was lower than at 6 hours JA elicitation. At the later time-points, loganic acid levels in both elicited and control cells were not different. The central intermediate of TIA, strictosidine, was about twofold increased at 24 and 72 hours after elicitation, while the serpentine level was only increased at the latter time-point. The tabersonine level was also increased (threefold) at 72 hours after elicitation. Overall, loganic acid concentration was increased between 6 – 24 hours after elicitation and subsequently the levels of strictosidine and the other down-stream TIAs gradually increased in time.

It is known that the increased levels of TIA upon jasmonate elicitation results from elevated expression of several genes encoding enzymes involved in TIA biosynthesis (Memelink et al. 2001). The jasmonate-responsive expression of a number of these biosynthesis genes is controlled by transcriptional regulators known as octadecanoid-derivative responsive *Catharanthus* AP2-domain (ORCA) proteins (Memelink et al. 2001). ORCA3 was shown to regulate the expression levels of *DXS*, *ASα*, *TDC*, *CPR*, *STR*, and *D4H* but not of *G10H* (or also known as *G8O*), *SGD*, and *DAT* (van der Fits and Memelink 2000). Another AP2/ERF-domain transcription factor called ORCA2 was not fully characterized but it was found to affect the expression of *STR* and *TDC* (Menke et al. 1999). ORCA2 and ORCA3 have been suggested to regulate different but overlapping sets of genes (Memelink et al. 2001). A study by Hasnain et al. (2010) showed that *ASα*, *TDC*, *SLS*, *STR*, and *D4H* genes were upregulated by both ORCA2 and ORCA3.

It should be noted that the composition of strictosidine, serpentine, and tabersonine in the control CRPP cell line at the 72 hour time-point was 93.3%, 6.1%, and 0.5% respectively, whereas after elicitation, those compounds were all increased but the relative composition was not really changed with strictosidine (93%), serpentine (6%), and tabersonine (1%). This shows that the flux through the pathway is increased, but within the TIA biosynthetic network the enzyme activities remain the same for the enzymes involved in the biosynthesis of serpentine and tabersonine, and apparently the activities are not fully used. This fits with the observations of Whitmer et al. (2002) who showed that by feeding TIA precursors the TIA network has a large overcapacity that under normal condition is not used.

Analysis of carotenoids

Carotenoids belong to the tetraterpenoids (C40) group and are derived from GGPP formed from the C5 precursors of the MEP pathway localized in the plastids. The key step of carotenoid biosynthesis is the formation of lycopene which is derived from phytoene after condensation of two GGPP molecules. Lycopene is a branch point for the production of two

cyclic carotenoids, *α*-carotene and *β*-carotene. Lutein derives from *α*-carotene, while *β*-carotene produces oxygenated derivatives of zeaxanthin, violaxanthin, and neoxanthin (Namitha and Negi 2010).

An increase level of carotenoids can be observed at 72 hours after JA elicitation in the *C. roseus* cell suspension culture (**Fig. 3**). At that time-point, the concentrations of all measured carotenoids in this study were about twofold higher in the JA-treated cells compared to the control. In addition, the levels of chlorophyll a and b were also increased after 72 hours in JA-treated cells (**Fig 4**). The composition of the mixture of carotenoids in the *C. roseus* cell culture did not alter after JA treatment; *β*-carotene stands for the largest part of the total carotenoids followed by lutein, neoxanthin, and violaxanthin.

Previous studies showed that jasmonate regulates the biosynthesis of carotenoids (Saniewski and Czapski 1983; Pérez et al. 1993; Rudell and Mattheis 2002; Liu et al. 2012). Jasmonate elicitation increased the expression level of a number of biosynthesis genes including *DXS* (van der Fits and Memelink 2000), encoding an enzyme of upstream part of the MEP pathway. Overexpressing the *DXS* gene in transgenic *Escherichia coli* resulted in an increased accumulation of the carotenoids lycopene (Harker and Bramley 1999; Matthews and Wurtzel 2000), *β*-carotene (Albrecht et la. 1999) or zeaxanthin (Albrecht et la. 1999; Matthews and Wurtzel 2000). Furthermore, the *GGPPS* transcript levels in *Taxus canadensis* cell suspension cultures (Hefner et al. 1998) and *Corylus avellana* leaves (Wang et al. 2010) were up-regulated after MeJA treatment, even though no significant change in *GGPPS* expression was observed in *C. roseus* cell suspension cultures (Thabet et al. 2012). Thabet et al. (2012) speculated that there are several homologues of GGPPS in *C. roseus* and only some of them may be induced by MeJA as exemplified in tomato (*Lycopersicon esculentum*), in which *LeGGPPS1* was induced by jasmonate acid but not the *LeGGPPS2* (Ament et al. 2006).

Carotenoids are essential in photosynthesis to absorb light energy and protect chlorophylls from photo damage (Namitha and Negi 2010). Pérez et al. 1993 showed that MeJA stimulates chlorophyll degradation in ‘Golden Delicious’ apple peel and chlorophyll a/b ratio decreases with increasing MJ exposure. In contrast, Rudell and Mattheis (2002) found a significant increase of chlorophyll b, while the level of chlorophyll a was relatively stable after MeJA treatment in ‘Fuji’ apples. Rudell and Mattheis (2002) suggested that the different results are probably due to the light treatment which was not applied by Pérez et al. (1993). In agreement with Rudell and Mattheis (2002), a notable increase of both chlorophylls a and b were observed at 72 hours after JA elicitation in our *C. roseus* cell

suspension cultures under light treatment. The chlorophyll a and b were increased in our study, this maybe the result of elevated activity of light harvesting connected with the increased level of carotenoids at that time-point (**Fig. 3** and **4)**.

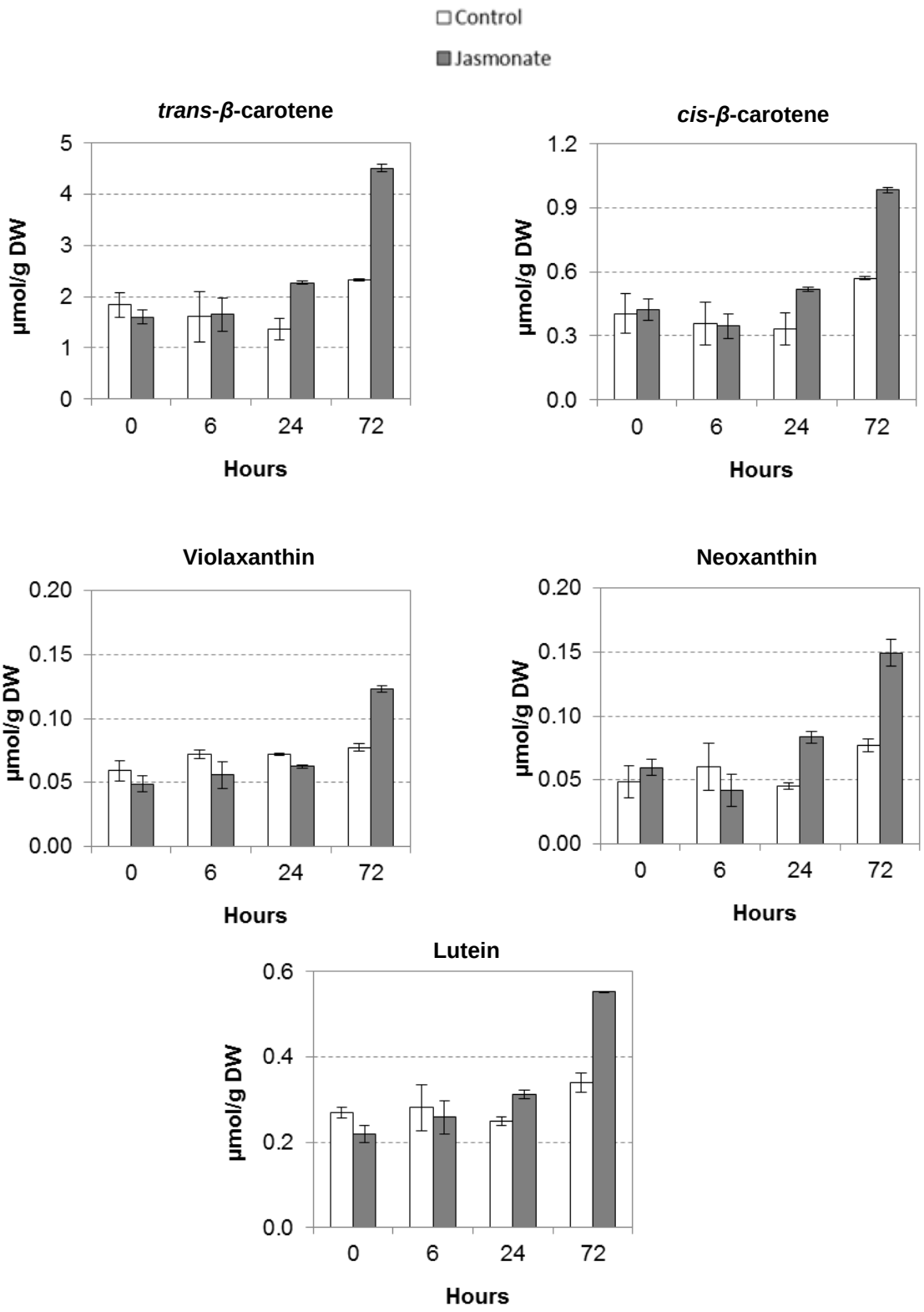


Fig. 3 Time course of carotenoid production in CRPP cell line elicited with 34 µM jasmonic acid or control solution measured by HPLC-Diode Array Detector (DAD). Results are the mean of two replicates; error bars indicate the two values.

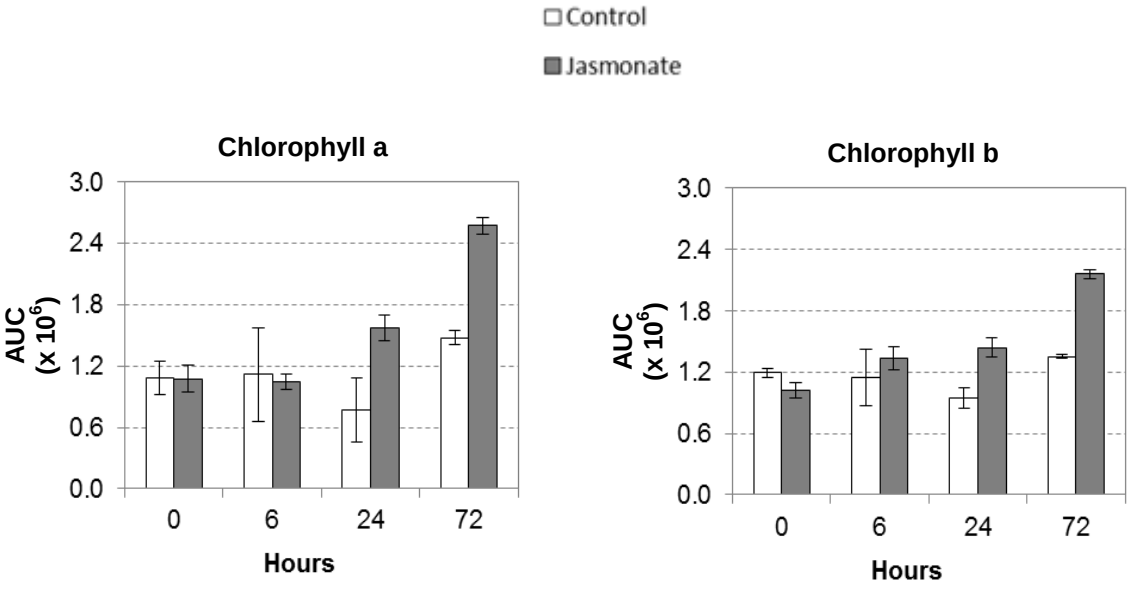


Fig. 4 Chlorophyll levels in CRPP cell line elicited with 34 µM jasmonic acid or control solution measured by HPLC-Diode Array Detector (DAD). Results are the mean of two replicates; error bars indicate the two values. AUC is area under the curve value.

Analysis of phytosterol

The levels of campesterol, *β*-sitosterol, and stigmasterol were not relatively different between JA-elicited and control cells (**Fig. 5)**. This indicates that JA elicitation at the concentration applied did not alter the biosynthesis of phytosterols. Studies in *Glycyrrhiza glabra* cell cultures (Hayashi et al. 2003) and the adventitious root cultures of *Panax ginseng* (Lee et al. 2004) showed that MeJA treatment did not induce the transcript level of cycloartenol synthase, the enzyme responsible for the first step in sterol biosynthesis. However, several studies showed contradictory results on phytosterols accumulation after MeJA elicitation in different plants or plant cell cultures. For example, phytosterols production was enhanced in *Lemna paucicostata* cultured plants (Suh et al. 2013) and *Capsicum annuum* cell culture (Sabater-Jara et al. 2010), decreased in *Centella asiatica* and *Ruscus aculeatus* cultured plants (Mangas et al. 2006), and remained constant in *Galphimia glauca* cultured plants (Mangas et al. 2006) and *Glycyrrhiza glabra* cell cultures (Hayashi et al. 2003). The different results may indicate the existence of different control mechanisms among the plant species.

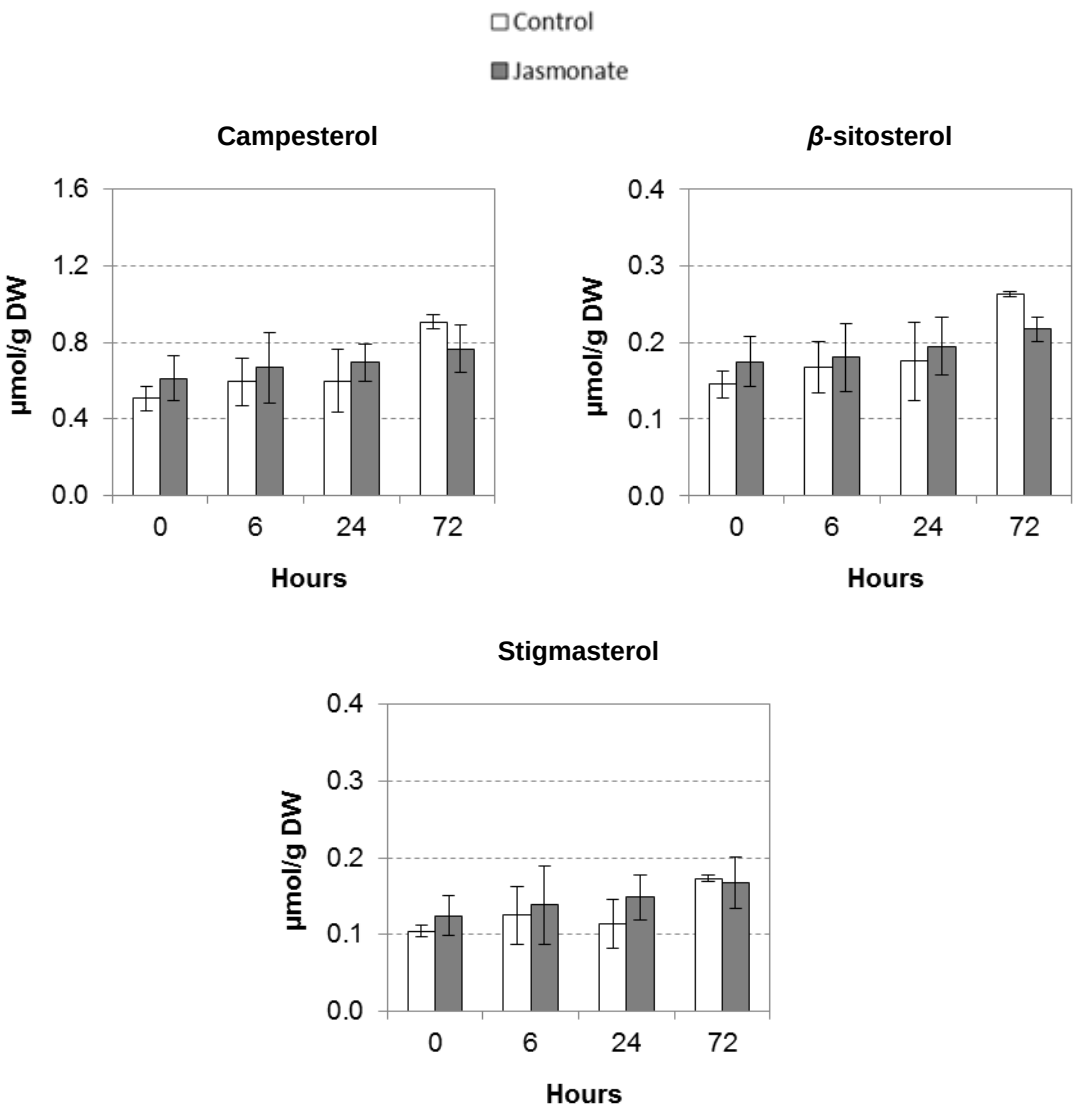


Fig. 5 Time course of sterols production in CRPP cell line elicited with 34 µM jasmonic acid or control solution measured by GC-Flame Ionization Detector (FID). Results are the mean of two replicates; error bars indicate the two values.

Total accumulation and C5 distribution

The effect of JA elicitation in terms of total accumulation of the various terpenoid groups and the distribution of five-carbon precursors (C5) was evaluated. In terms of total accumulation of the terpenoid groups, monoterpenoids consisting of loganic acid and several TIAs were highly accumulated during the growth time-course and after elicitation compared to carotenoids and sterols (**Fig. 6a).** The major contribution (82%) to the total composition of monoterpenoid accumulation comes from strictosidine, which indicates that the flux towards strictosidine is highly active in this specific *C. roseus* cell line. Total phytosterols content was relatively constant, whereas total carotenoids level was increased at 24 and 72 hours after JA

elicitation. These results suggest a relationship between TIA and carotenoid pathways induction as can be explained by up-regulation of several genes encoding enzymes in the MEP, TIA, and carotenoid pathways upon jasmonate elicitation (Thabet et al. 2012; van der Fits and Memelink 2000).

As regards to the distribution of C5 precursors, the level of C5 units increased in both carotenoids and TIA after 24 and 72 hours of elicitation, whereas phytosterols did not change compared to the control (**Fig. 6b**). This shows that upon jasmonate elicitation, the carbon flow is activated towards the MEP pathway rather than the MVA pathway leading to phytosterols, and that export of IPP::DMAPP intermediates to the cytosolic terpenoid routes like the phytosterol is minimal. At 24 hours after elicitation, the level of the C5 unit in TIAs was 1.8-fold higher in elicited cells (15.86 µmol/g DW) compared to the control (8.80 umol/g DW), whereas in carotenoids a 1.6-fold increase of C5 units was observed in the elicited cells (25.97 umol/g DW) compared to the control (16.45 umol/g DW). Subsequently, after 72

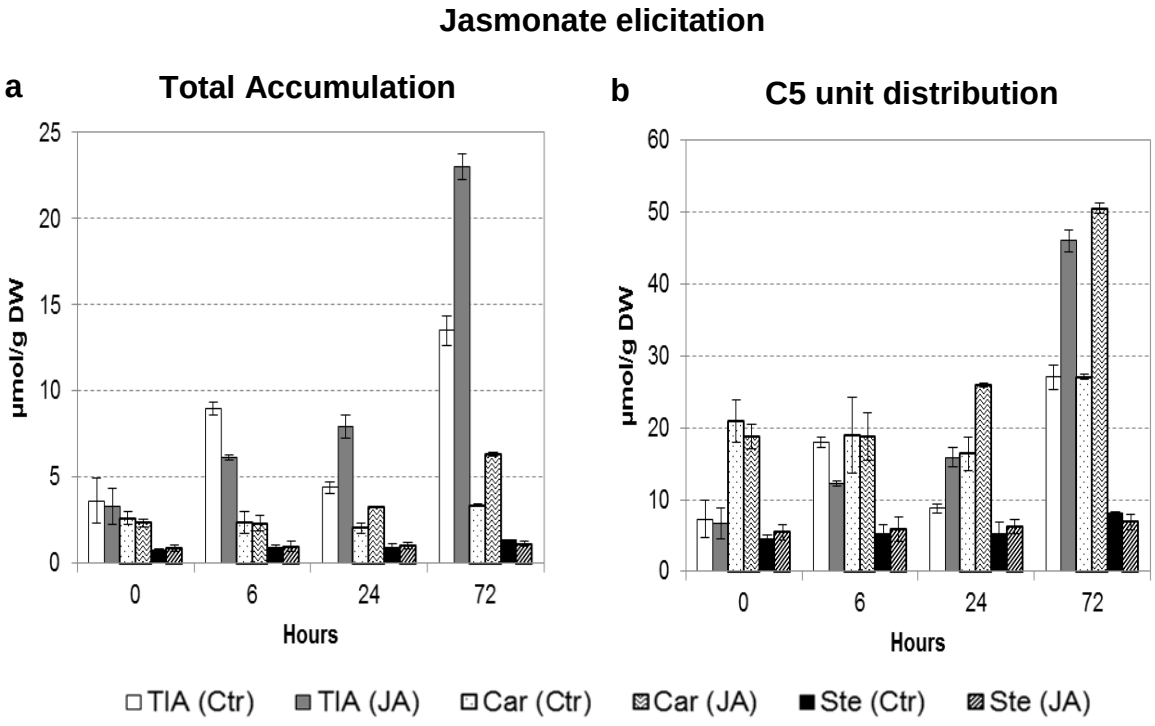


Fig. 6 Total accumulation (a) of terpenoid indole alkaloids, carotenoids, and sterol and the C5 precursor distribution (b) after jasmonic acid elicitation (34 µM) in the CRPP cell line. Results are the mean of two replicates; error bars indicate the two values.

hours of elicitation, the distribution of the C5 unit to these pathways was almost doubled and interestingly between and within these pathways the ratios of the compounds more or less remain the same as in the control. Pointing at a regulation at the very beginning of the MEP pathway producing more C5 units which are then, by the existing biochemical machinery converted to the various products resulting in similar ratios of these as in control cells. The results suggest that both carotenoid and TIA pathways benefit from an increased carbon flow in the MEP pathway, but that elicitation does not really change the carbon flux distribution between the two pathways. TIA production might be further increased by changing the C5 distribution into these pathways.

Metabolite profiles using NMR

The extracts of *C. roseus* cell suspension cultures harvested at different time-points after JA treatment and controls were subjected to ^{1}H-NMR analysis. **Fig 7** shows the comparison of ^{1}H-NMR spectra of JA-elicited and control samples after 72 hours treatment. The amino acids isoleucine, leucine, valine, and alanine were identified. Organic acids like acetic acid, succinic acid, malic acid, fumaric acid, and formic acid were also found. In the carbohydrate region, signals of anomeric protons of β-glucose, α-glucose, and sucrose were detected. The aromatic part of the ^{1}H-NMR spectra showed signals of indole moiety (twin triplets and doublets around δ 7.00 – δ 7.50) and the presence of signals at δ 7.07 (*t*, J = 7.5 Hz, H-10), δ 7.16 (*t*, J = 7.5 Hz, H-11), δ 7.37 (*d*, J = 8.0 Hz, H-12), δ 7.49 (*d*, J = 8.0 Hz, H-9), δ 7.80 (*s*, H-17), and δ 5.80 (*d*, J = 8.5 Hz, H-1') represent characteristic signals of strictosidine. The other compound detected is loganic acid by the presence of signals at δ 1.08 (*d*, J = 6.7 Hz, H-10), δ 4.68 (*d*, J = 8.0 Hz, H-7), δ 5.27 (*d*, J = 3.5 Hz, H-1), δ 7.08 (*s*, H-3). The assignments of ^{1}H-NMR signals were performed by comparing the reference spectra of our in-house database and the results of previous reports (Kim et al. 2006, 2010; Mustafa et al. 2009; Saiman et al. 2012).

The NMR method developed in our group has been successfully applied for metabolite analysis in plant extracts (Kim et al. 2010). It is a robust method for metabolic analysis particularly in terms of reproducibility. NMR methods offer some advantages compared to conventional chromatography or MS methods. The range of compounds is less limited by their polarity and specific chemical features such as chromophores, which means that a broader range of metabolites can be detected by NMR. The NMR extraction method is simple and fast. The solvent used can be adjusted for analysis of a particular target group. However, NMR detection may be less suitable for compounds present at relatively low levels

using the usual short time of sample analysis. Nevertheless, this limitation can be improved considerably by increasing the number of scans or using higher field strength. In addition, the application of cryoprobes and microprobes has greatly contributed to increase the NMR sensitivity (Kim et al. 2006, 2010). Two-dimensional NMR analysis can be applied to confirm the presence of a compound in the extract as well as to overcome in part the problem of overlapping signals.

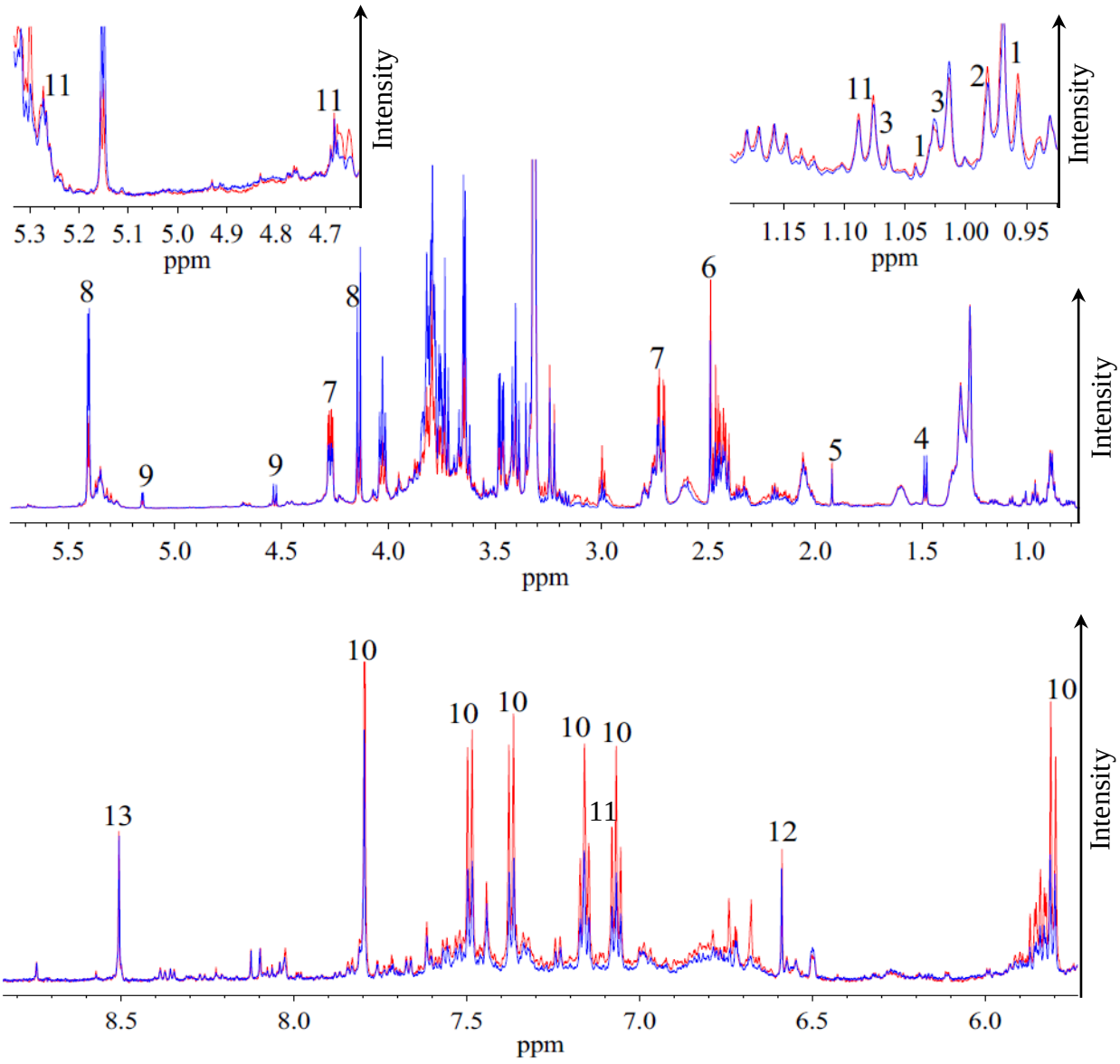


Fig. 7 ^{1}H-NMR spectra of jasmonate-elicited (red) and control (blue) cell suspension cultures of *Catharanthus roseus* at 72 hours. 1: isoleucine, 2: leucine, 3: valine, 4: alanine, 5: acetic acid, 6: succinic acid, 7: malic acid, 8: sucrose (fructose moiety: δ 4.14, glucose moiety: δ 5.41), 9: glucose (β-glucose: δ 4.53, α-glucose: δ 5.15), 10: strictosidine, 11: loganic acid, 12: fumaric acid, 13: formic acid.

Multivariate data analysis of NMR data

To analyze the changes in the metabolic profiles between JA-elicited and control cells of *C. roseus*, chemometric methods were applied. Principal component analysis (PCA) is the most common method used to analyze data that arises from more than one variable. PCA is an unsupervised clustering method requiring no knowledge of the data set, which is used to reduce the dimensionality of multivariate data and displays systemic variation between the samples in an unbiased way (Eriksson et al. 2001, 2006). In this study, PCA was applied to the bucketed data of ^{1}H-NMR spectra. The chemical shifts (ppm) were used as variables and the samples served as observations in the data matrix. The data was mean-centered and variables were scaled to the Pareto method. The results are presented in a graphical form known as score plot, which differentiates samples based on the variables. The loading plot is used to find variables, which contribute to separation of the samples.

Figure 8 presents the PCA score plot of the samples. The plot shows that the samples were separated due to the growth cycle, in which the older samples moved to the negative part of PC1. This indicates that the metabolites profiles change with the developmental status of the cells. The PCA did not exhibit a clear distinction at 0 – 6 hours after elicitation but a separation of the elicited and control samples was observed beyond 24 hours after elicitation. PCA is an unbiased method which shows maximum variation within the samples. If the biological variation among the replicates is larger than between the groups, a clear separation is not observed. This was in fact found here for the early time-point samples and as experienced by Ali et al. (2012) in infected and control samples of grapevine at different time intervals. An effect of the developmental stage on the metabolome was also found in our *C. roseus* cell-line A12A2 when studying the time-effect associated to salicylic acid elicitation (Mustafa et al. 2009).

To extract information about specific variables, a supervised multivariate analysis is needed. Thus, partial least squares-discriminant analysis (PLS-DA) was applied to the same bucketed ^{1}H-NMR spectra. In addition to the X-matrix of NMR data, two classes (JA-treated and control cell) were assigned for the Y-matrix in PLS-DA. The variables were mean-centered and scaled to unit variance. However, PLS-DA was unable to give any significant model that fitted to the data. It was decided to separate the samples into two groups; 0 – 6 hours and 24 – 72 hours since the earlier result in PCA shows such separation between JA elicitation and control samples beyond 24 hours of elicitation. As expected, the PLS-DA of the samples at 0 – 6 hours time intervals did not deliver any fit component model, which

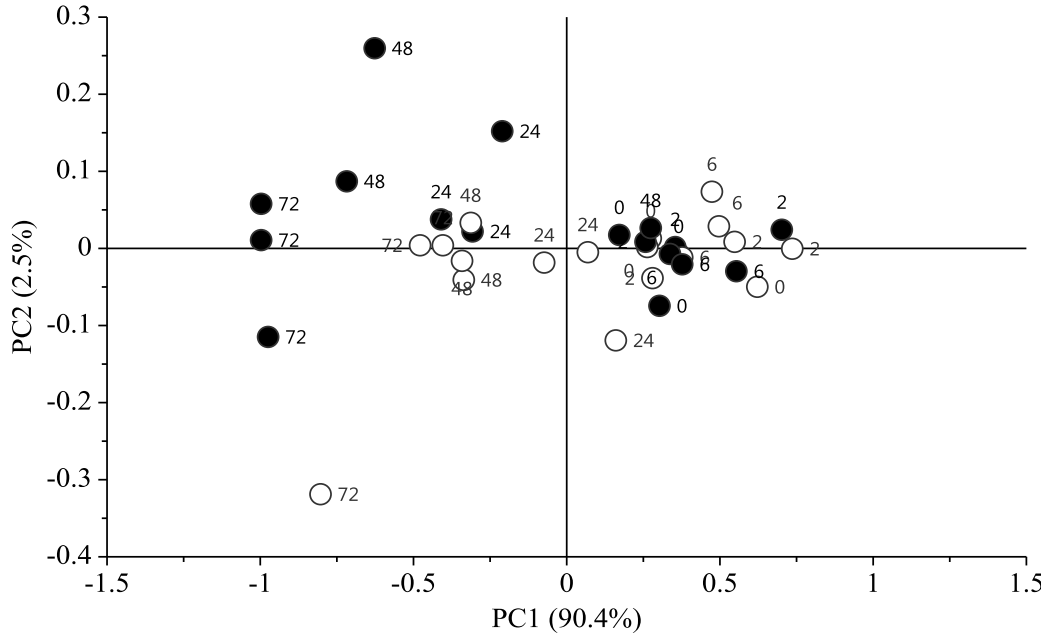


Fig. 8 Score plot of principal component analysis (PCA) of jasmonic acid-elicited (●) and control (○) samples of the CRPP cell line measured by ^{1}H-NMR. The numbers in the score plot are harvesting time (hour) after treatments.

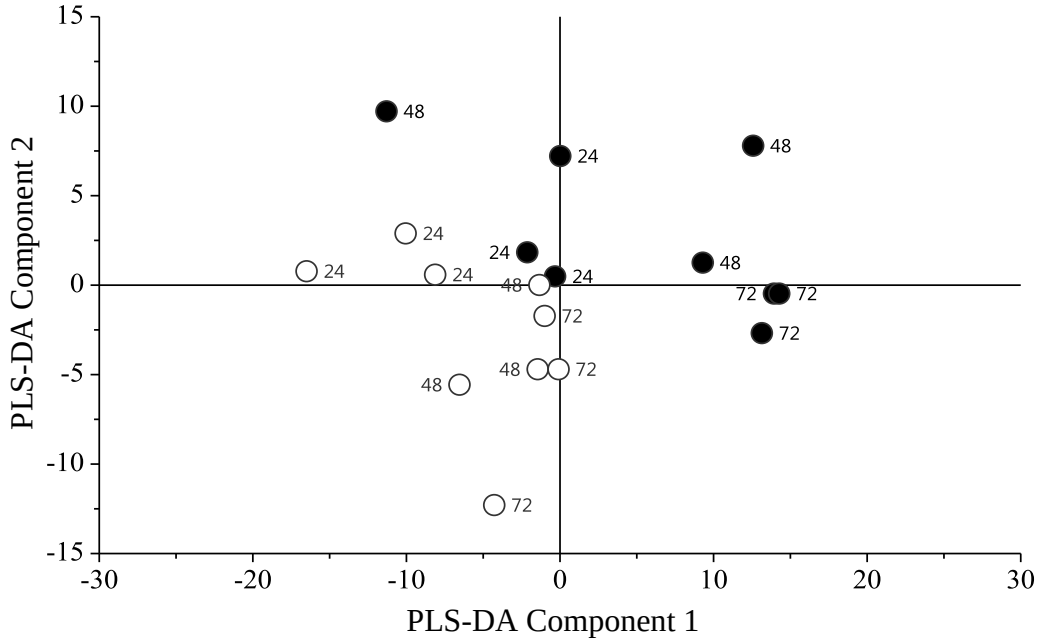


Fig. 9 Score plot of partial least squares-discriminant analysis (PLS-DA) of jasmonic acid-elicited (●) and control (○) samples of the CRPP cell line measured by ^{1}H-NMR. The numbers in the score plot are harvesting time (hour) after treatments.

indicates no significant separation among the samples. Instead, the samples at 24 – 72 hour time-points gave five component models with R2 and Q2 values of 0.95 and 0.79, respectively. These PLS-DA models were validated by the permutation method through 100 applications in which the Q2 values of permuted Y vectors were lower than the original ones and the regression of Q2 lines intersect at below zero. The score plot in **Fig. 9** shows a separation between JA-treated and control samples observed at 24 – 72 hours after elicitation. There is one outlier observed for the JA-treated samples after 48 hours elicitation, which distantly separates from its biological replicates. This could be due to either technical or biological variation of the sample, in which the latter is the most influential in many cases.

To get a better separation of the validated PLS-DA model, another supervised algorithm, orthogonal projection to latent structures-discriminant analysis (OPLS-DA) was applied to the same data. OPLS-DA is used as improvement of the PLS-DA method to separate two or more classes of multivariate data (Bylesjö et al. 2006; Westerhuis et al. 2010). In OPLS-DA, a regression model is calculated between the multivariate data and a response variable that only contains class information (Westerhuis et al. 2010). The advantage of OPLS-DA compared to PLS-DA is that a single component is used as a predictor for the class, while the other components describe the variation orthogonal to the first predictive component (Westerhuis et al. 2010). As shown in **Fig. 10,** OPLS-DA clearly distinguished between JA-treated and control samples after 24 – 72 hours of elicitation. The loading plot (**Fig. 11**) reveals that the JA-elicited cells were higher in aromatic and amino/organic acid regions, while the control cells were distinguished by sugars. Some of the compounds identified for the separation of JA-elicited cells were strictosidine, succinic acid, fumaric acid, malic acid, and leucine. Sucrose, glucose, and alanine were found higher in the control samples. The increased level of strictosidine and decreased level of sugars in JA-elicited cells suggest that sugars are used for the production of ATP and as carbon sources of plant defense compounds.

Instead of using PLS-DA, PLS can also be used to reduce the dimensionality of multivariate data and to find relations between X and Y matrices. In PLS-DA, a dummy Y-variables is created by assigned classes, while in PLS, additional data are included as Y-data sets; in this case time and elicitation. As for the PLS-DA, all samples from 0 – 72 hour time-points did not deliver a validated PLS model, whereas only the 24 – 72 hours samples deliver a good PLS model (**Fig. 12)**. Since the PLS has more than one Y-variable, bidirectional orthogonal PLS (O2PLS) was used in order to discriminate the cells by time and treatment.

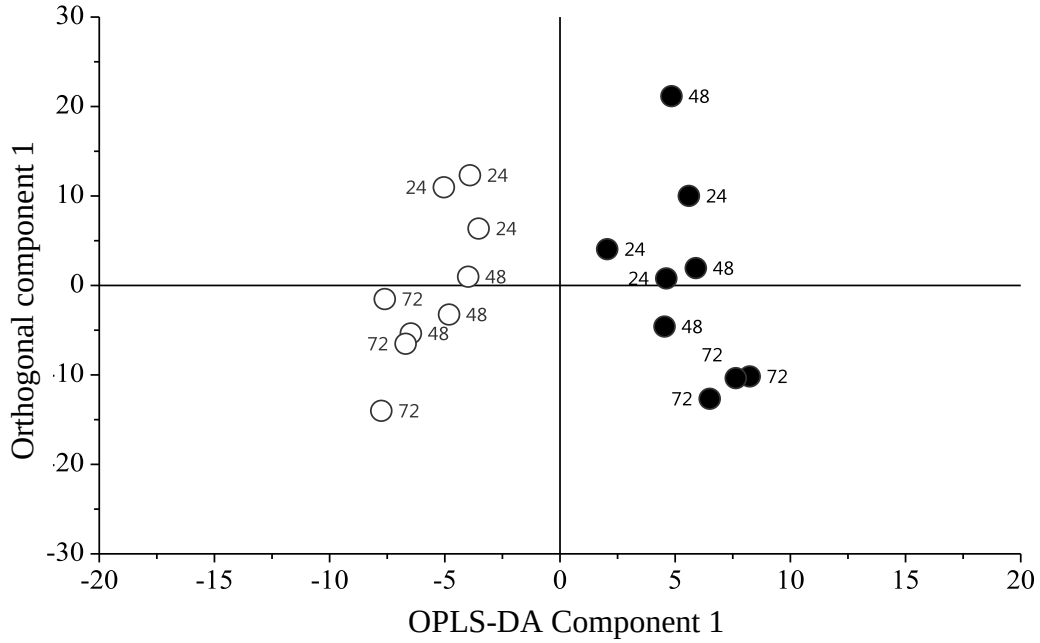


Fig. 10 Score plot of orthogonal projection to latent structures-discriminant analysis (OPLS-DA) of jasmonic acid-elicited (●) and control (○) samples of the CRPP cell line measured by ^{1}H-NMR. The numbers in the score plot are harvesting time (hour) after treatments.

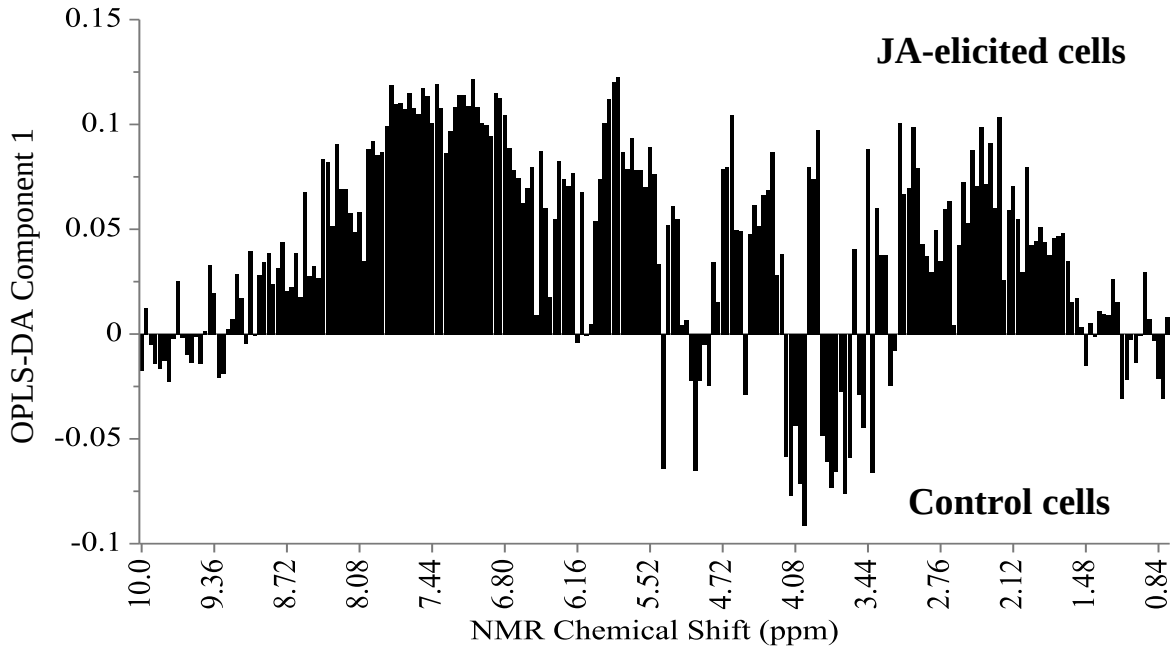


Fig. 11 Loading plot of orthogonal projection to latent structures-discriminant analysis (OPLS-DA) of jasmonic acid (JA)-elicited CRPP cells and control cells.

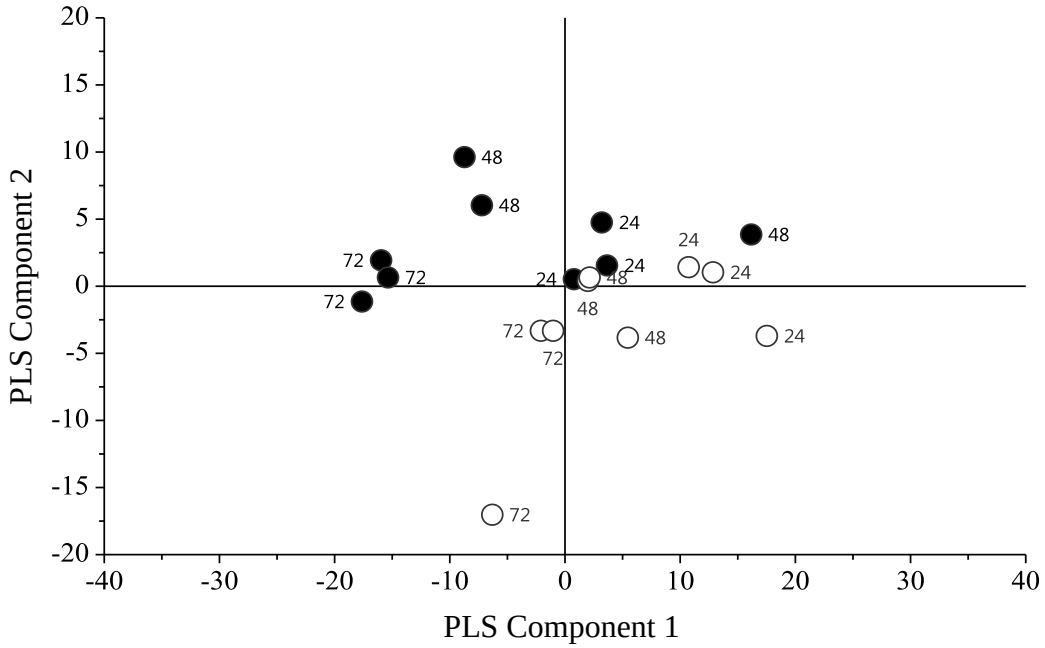


Fig. 12 Score plot of partial least squares (PLS) of jasmonic acid-elicited (●) and control (○) samples of the CRPP cell line measured by ^{1}H-NMR. The numbers in the score plot are harvesting time (hour) after treatments.

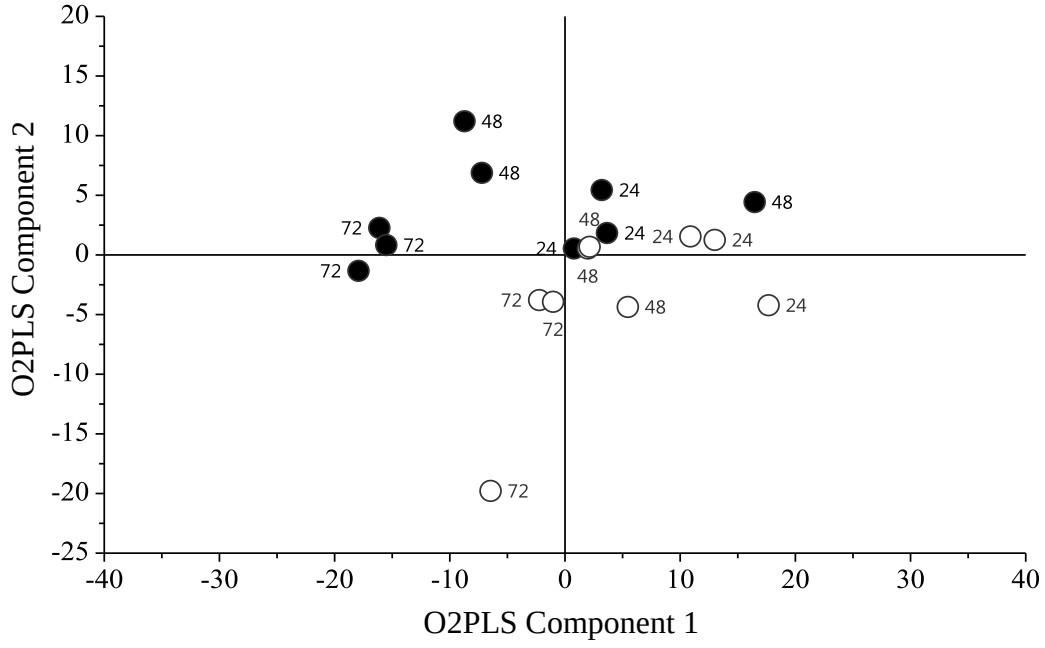


Fig. 13 Score plot of bidirectional orthogonal projection to latent structures (O2PLS) of jasmonic acid-elicited (●) and control (○) samples of the CRPP cell line measured by ^{1}H-NMR. The numbers in the score plot are harvesting time (hour) after treatments.

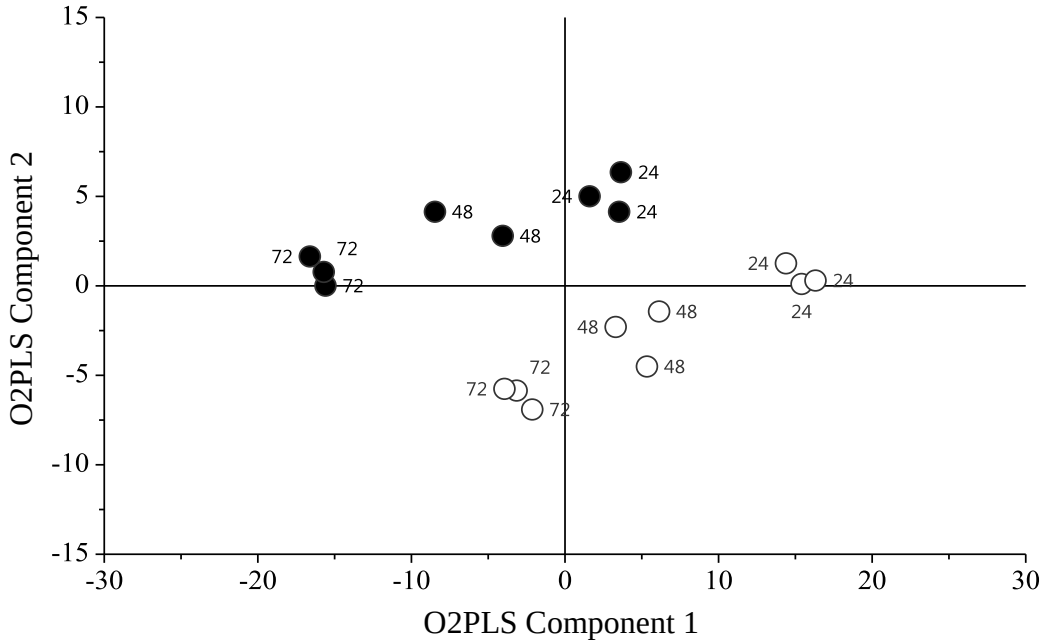


Fig. 14 Score plot of bidirectional orthogonal projection to latent structures (O2PLS) shows better separation of jasmonic acid-elicited (●) and control (○) samples after removing an outlier of JA-elicited sample at 48 hours. The numbers in the score plot are harvesting time (hour) after treatments.

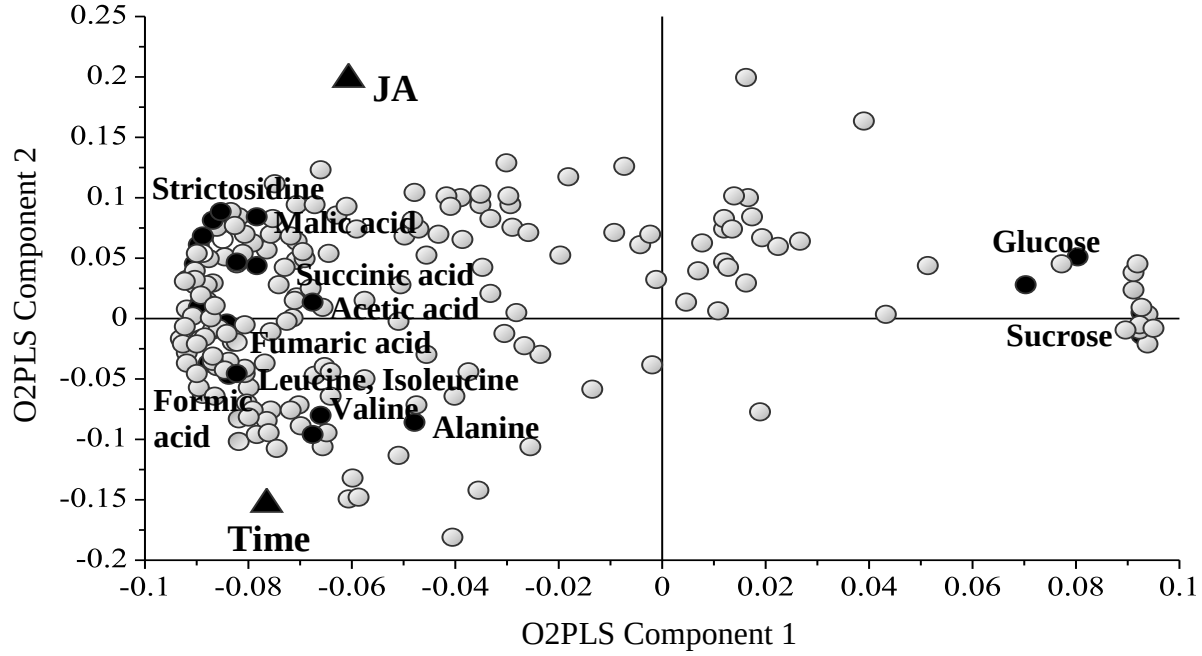
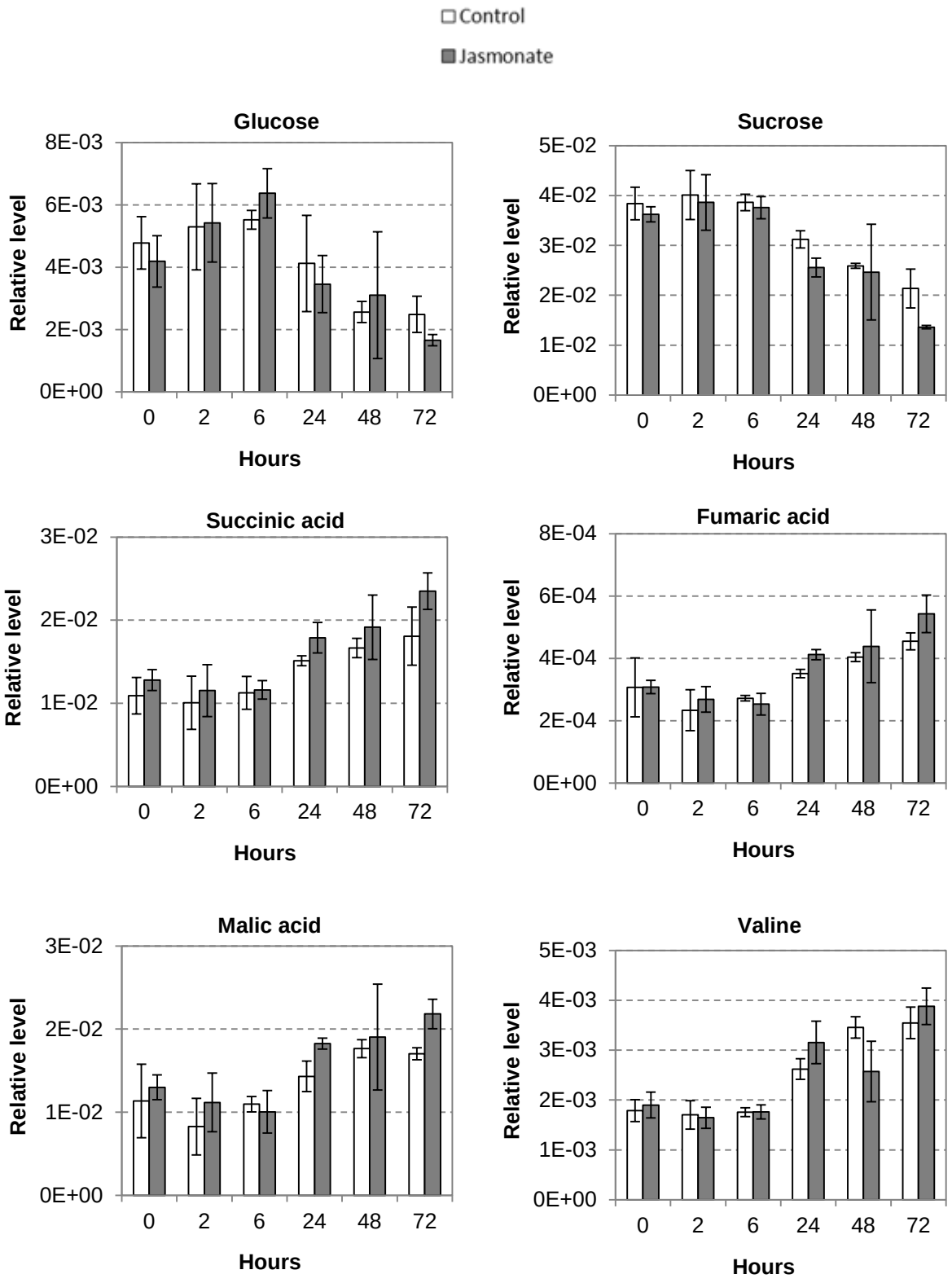


Fig. 15 Loading plot of bidirectional orthogonal projection to latent structures (O2PLS) shows relation of variables based on jasmonic acid (JA) treatment and cell age (time).

Nevertheless, the sample separation in O2PLS showed a similar pattern as in the PLS model **(Fig. 13)**. By removing the forementioned outlier of the 48-hours JA-elicited samples, the O2PLS model was improved **(Fig. 14).** The O2PLS loading plot (**Fig. 15**) shows that most of the metabolites were relatively increased through time except sugars. Strictosidine, malic acid, and succinic acid were most influenced by JA elicitation over the time period.

Relative levels of metabolites detected by NMR

Although multivariate data analysis, either supervised or unsupervised, is useful to reduce the dimensionality of a multivariate dataset and to identify differences or similarities among the samples, the time-course levels of individual metabolites involved in the samples discrimination are not presented. **Figure 16** shows the transient levels of several compounds identified from their characteristic signal in NMR data. As revealed in OPLS-DA and O2PLS results, sugars levels decreased by time and sucrose levels were significantly lower ($P < 0.05$) at 24 and 72 hours in the JA-elicited cells compared to the control. In contrast, Flores-Sanchez et al. (2009) found no significant difference of sucrose and glucose levels in methyl jasmonate-elicited *Cannabis sativa* cell suspension cultures if compared to their respective controls. Some components of the TCA cycle; succinic acid, fumaric acid, and malic acid were increased at 24 and 72 hours after elicitation, although only malic acid levels were statistically significant compared to the control ($P < 0.05$). A similar result was found in the cell suspension cultures of *C. sativa* in which fumaric acid level was induced after MeJA elicitation (Flores-Sanchez et al. 2009). The current results suggest that upon jasmonate elicitation, sugars were catabolized and TCA cycle was activated to make building blocks and generate chemical energy for various biosynthetic routes that are part of the defense response. The levels of valine remained statistically unaltered between JA-elicited and control cells at all time-points ($P < 0.05$). Leucine was found higher at 24 and 72 hour after elicitation in which the level at the former time-point was significantly increased ($P < 0.05$). Interestingly, the amino acid alanine was significantly decreased after 72 hour of elicitation ($P < 0.05$), which might be linked to the decreased levels of sugars. Liang et al. (2006) also found that glucose, sucrose, and alanine were decreased in MeJA treated *Brassica rapa* leaves compared to the control. In agreement to HPLC results, strictosidine levels were significantly increased at 24 and 72 hours after elicitation ($P < 0.05$). The level of loganic acid in JA-elicited cells increased at 24 hours ($P < 0.05$), whereas loganic acid levels were not significantly different between elicited and control cells at 72 hours after elicitation.



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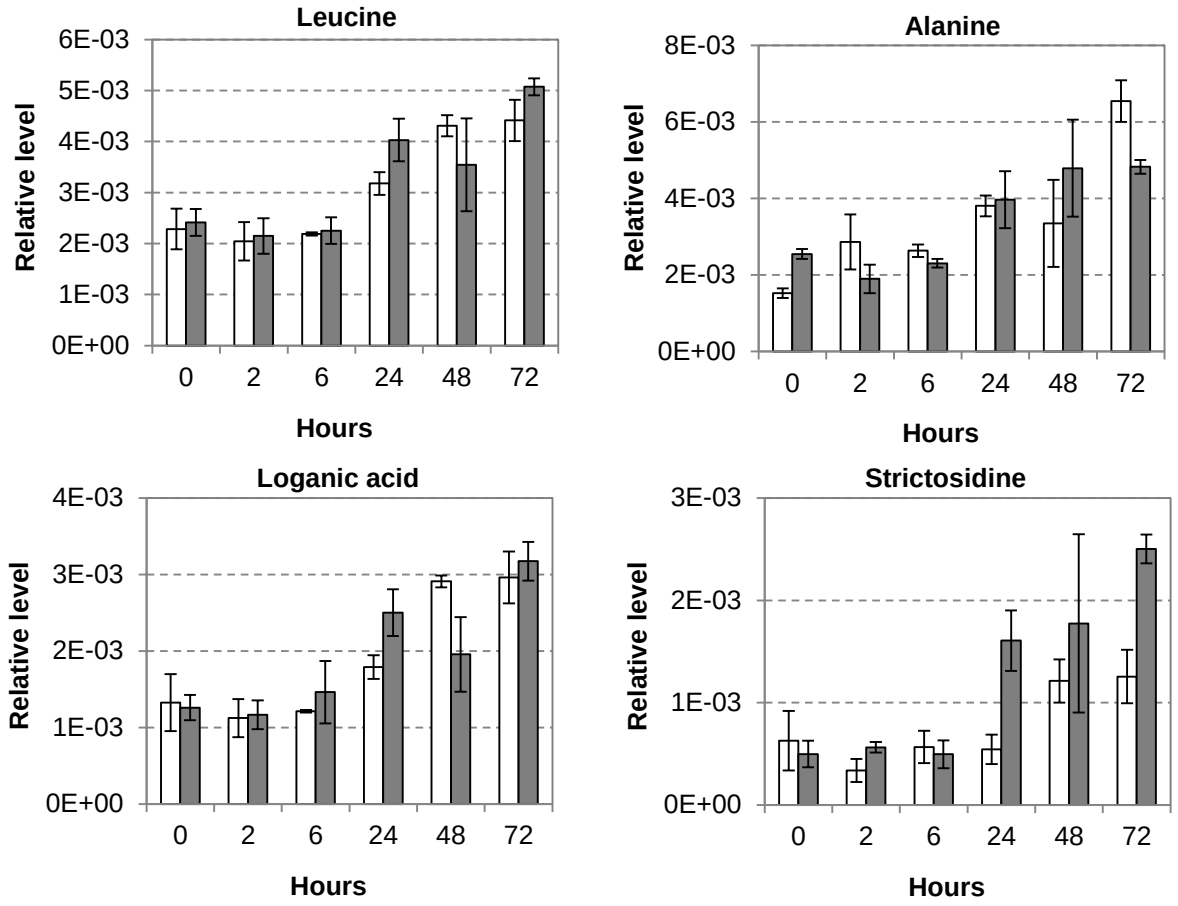

Fig. 16 Relative level of metabolites based on the mean area (^{1}H-NMR bucket data) of the resonance peak related to the metabolites. Results are average of three replicates ± standard deviation.

Conclusion

The effects of JA elicitation on primary and secondary metabolism in *C. roseus* cell suspension cultures were studied. **Figure 17** summarizes the metabolic effect of JA elicitation in this study. Elicited cells showed higher levels of TIA and carotenoids, while sterol accumulation was not affected, indicating that the flux of C5 units was only increased in the MEP pathway upon JA elicitation. For both carotenoid and TIA pathway, the available enzymatic machinery seems to have a large overcapacity, able to deal with in increased carbon flux from the MEP pathway. To increase TIA production, channeling the increased availability of the C5 units away from carotenoids to TIA would be an interesting strategy. This shows that a systemic study of the metabolic changes in cells can lead to novel approaches of metabolic engineering.

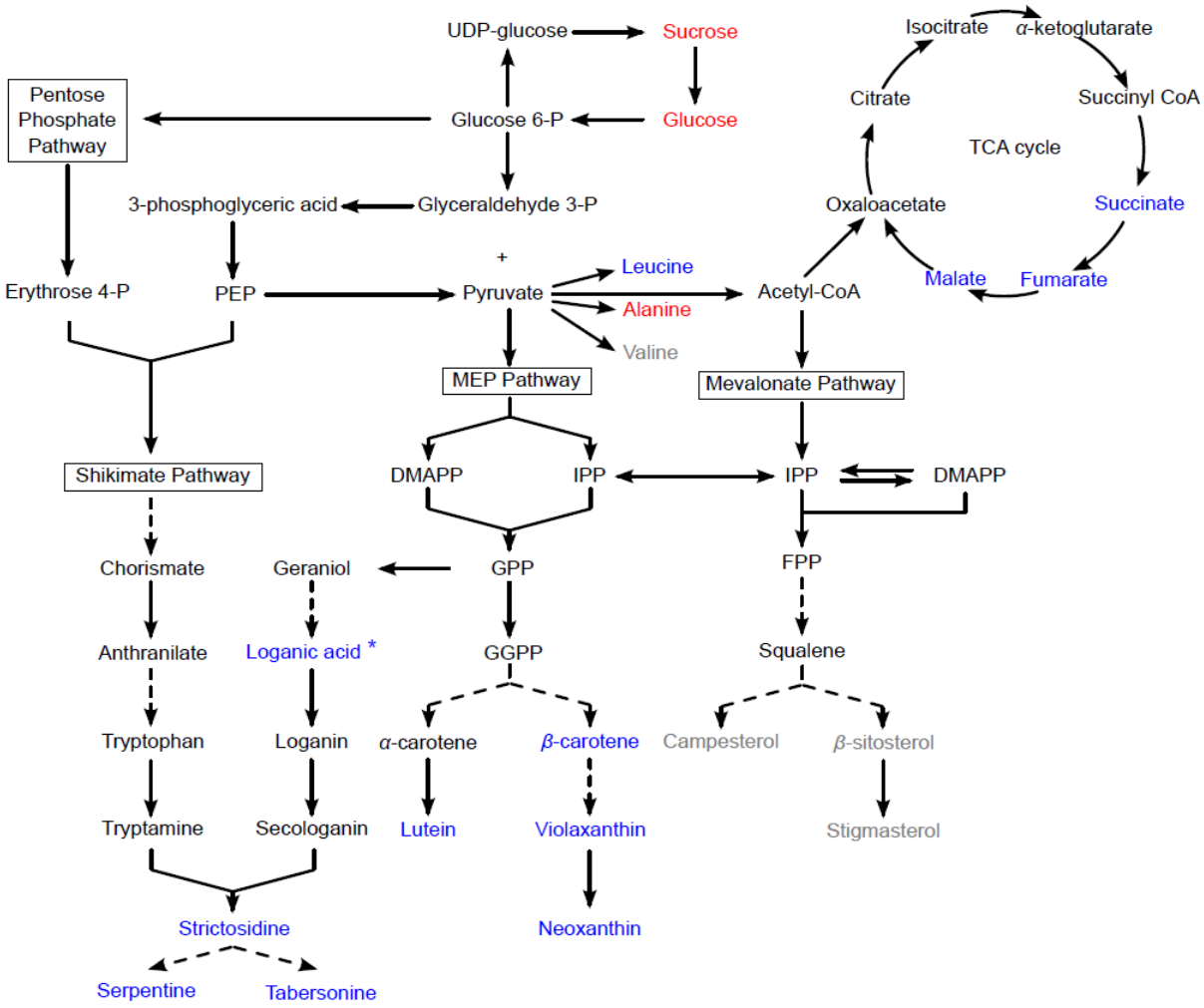

Fig. 17 Schematic summary of the results obtained in this study. The levels of the identified metabolites which were increased (blue), decreased (red), or relatively unchanged (grey) after jasmonic acid elicitation in *C. roseus* cell suspension cultures. *Loganic acid level increased at 24 hours after elicitation, whereas at 72 hours the level is about similar to the level of the control.

Acknowledgements

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References

Albrecht M, Misawa N, Sandmann G (1999) Metabolic engineering of the terpenoid biosynthetic pathway of *Escherichia coli* for production of the carotenoids *β*-carotene and zeaxanthin. Biotechnol Lett 21: 791–795

Ali K, Maltese F, Figueiredo A, Rex M, Forte AM, Zyprian E, Pais MS, Verpoorte R, Choi YH (2012) Alterations in grapevine leaf metabolism upon inoculation with *Plasmopara viticola* in different time-points. Plant Sci 191–192: 100–107

Ament K, Van Schie CC, Bouwmeester HJ, Haring MA, Schuurink RC (2006) Induction of a leaf specific geranylgeranyl pyrophosphate synthase and emission of (E, E)-4, 8, 12-trimethyltrideca-1, 3, 7, 11-tetraene in tomato are dependent on both jasmonic acid and salicylic acid signaling pathways. Planta 224: 1197–1208

Bylesjö M, Rantalainen M, Cloarec O, Nicholson JK, Holmes E, Trygg J (2006) OPLS discriminant analysis: Combining the strengths of PLS-DA and SIMCA classification. J Chemometrics 20: 341–351

El-Sayed MA, Verpoorte R (2002) Effect of phytohormones on growth and alkaloid accumulation by a *Catharanthus roseus* cell suspension culture fed with the alkaloid precursors tryptamine and loganin. Plant Cell Tiss Organ Cult 68: 265– 270

El-Sayed MA, Verpoorte R (2004) Growth, metabolic profiling and enzymes activities of *Catharanthus roseus* seedlings treated with plant growth regulators. Plant Growth Reg 4: 53–58

Eriksson L, Johansson E, Kettaneh-Wold N, Wold S (2001) Multi- and Megavariate Data Analysis. Umetrics, Umeå, Sweden

Eriksson L, Johansson E, Kettaneh-Wold N, Tyrgg J, Wikström C, Wold S (2006) Multi- and Megavariate Data Analysis Part 1: Basic Principles and Applications, second ed. Umetrics, Umeå, Sweden

Flores-Sanchez IJ, Peč J, Fei J, Young Hae Choi YH, Dušek J, Verpoorte R (2009) Elicitation studies in cell suspension cultures of *Cannabis sativa* L. J Biotechnol 143: 157–168

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50: 155–158

Gundlach H, Müller MJ, Kutchan TM, Zenk MH (1992) Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. Proc Natl Acad Sci USA 89: 2389–2393

Harker M, Bramley PM (1999) Expression of prokaryotic 1-deoxy-D-xylulose-5-phosphatases in *Escherichia coli* increases carotenoid and ubiquinone biosynthesis. FEBS Lett 448: 115–119

Hasnain G (2010) The ORCA-ome as a key to understanding alkaloid biosynthesis in *Catharanthus roseus*. PhD Thesis, Leiden University, The Netherlands. Chapter 2, Genome-wide determination of ORCA target genes in *Catharanthus* alkaloids, pp. 28–48. ISBN 9789085705826

Hayashi H, Huang P, Inoue K (2003) Up-regulation of soya saponin biosynthesis by methyl jasmonate in cultured cells of *Glycyrrhiza glabra*. Plant Cell Physiol 44: 404–411

Hefner J, Ketchum RE, Croteau R (1998) Cloning and functional expression of a cDNA encoding geranylgeranyl diphosphate synthase from *Taxus canadensis* and assessment of the role of this prenyltransferase in cells induced for taxol production. Arch Biochem Biophys 360: 62–74

Hemmerlin A, Hoeffler JH, Meyer O, Tritsch D, Kagan IA, Grosdemange-Billiard C, Rohmer M, Bach TJ (2003) Cross-talk between the cytosolic mevalonate and the plastidial methylerythritol phosphate pathways in Tobacco Bright Yellow-2 cells. J Biol Chem 18: 26666 –26676

Kim HK, Choi YH, Verpoorte R (2006) Metabolomic analysis of *Catharanthus roseus* using NMR and principal component analysis. In: Saito K, Dixon RA, Willmitzer L (eds.) Biotechnology in Agriculture and Forestry, vol. 57, Plant Metabolomics, Springer, Leipzig, pp. 261–276

Kim HK, Choi YH, Verpoorte R (2010) NMR-based metabolomic analysis of plants. Nat Protoc 5: 536–549

Laule O, Fürholz A, Chang H-S, Zhu T, Wang X, Heifetz PB, Gruissem W, Lange BM (2003) Crosstalk between cytosolic and plastidial pathways of isoprenoid biosynthesis in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 100: 6866–6871

Lee MH, Jeong JH, Seo JW, Shin CG, Kim YS, In JG, Yang DC, Yi JS, Choi YE (2004) Enhanced triterpene and phytosterol biosynthesis in *Panax ginseng* overexpressing squalene synthase gene. Plant Cell Physiol 45: 976–984

Lee-Parsons CWT, Royce AJ (2006) Precursor limitations in methyl jasmonate-induced *Catharanthus roseus* cell cultures. Plant Cell Rep 25: 607–612

Liang YS, Choi YH, Kim HK, Linthorst HJM, Verpoorte R (2006) Metabolomic analysis of methyl jasmonate treated *Brassica rapa* leaves by 2-dimensional NMR spectroscopy. Phytochemistry 67: 2503–2511

Liu L, Wei J, Zhang M, Zhang L, Li C, Wang Q (2012) Ethylene independent induction of lycopene biosynthesis in tomato fruits by jasmonates. J Exp Bot 63: 5751–5761

Mangas S, Bonfill M, Osuna L, Moyano E, Tortoriello J, Cusido RM, Pinol MT, Javier Palazon J (2006) The effect of methyl jasmonate on triterpene and sterol metabolisms of *Centella asiatica*, *Ruscus aculeatus* and *Galphimia glauca* cultured plants. Phytochemistry 67: 2041–2049

Matthews PD, Wurtzel ET (2000) Metabolic engineering of carotenoid accumulation in *Escherichia coli* by modulation of the isoprenoid precursor pool with expression of deoxyxylulose phosphate synthase. Appl Microbiol Biotechnol 53: 396–400

Memelink J, Kijne JW, van der Heijden R, Verpoorte R (2001) Genetic modification of plant secondary metabolite pathways using transcriptional regulators. Adv Biochem Eng Biotechnol 72: 103–125

Menke FL, Champion A, Kijne JW, Memelink J (1999) A novel jasmonate- and elicitor-responsive element in the periwinkle secondary metabolite biosynthetic gene Str interacts with a jasmonate- and elicitor-inducible AP2-domain transcription factor, ORCA2. EMBO J 18: 4455–4463

Moreno PRH, van der Heijden R, Verpoorte R (1993) Effect of terpenoid precursor feeding and elicitation on formation of indole alkaloids in cell suspension cultures of *Catharanthus roseus*. Plant Cell Rep 12: 702-705.

Moreno PRH, van der Heijden R, Verpoorte R (1995) Cell and tissue cultures of *Catharanthus roseus*: a literature survey II. Updating from 1988 to 1993. Plant Cell Tiss Organ Cult 42:1–25

Moreno PRH, Poulsen C, van der Heijden R, Verpoorte R (1996) Effects of elicitation on different metabolic pathways in *Catharanthus roseus* (L.) G. Don cell suspension cultures. Enzyme Microb Technol 18: 99 –107

Mustafa NR, Kim Hye Kyong, Choi YH, Verpoorte R (2009) Metabolic changes of salicylic acid-elicited *Catharanthus roseus* cell suspension cultures monitored by NMR-based metabolomics. Biotechnol Lett 31: 1967–1974

Namitha KK, Negi PS (2010) Chemistry and biotechnology of carotenoids. Crit Rev Food Sci Nutr 50: 728–760

Pérez AG, Sanz C, Richardson DG, Olías JM (1993) Methyl jasmonate vapor promotes *β*-carotene synthesis and chlorophyll degradation in Golden Delicious apple peel. J Plant Growth Regul 12: 163–167

Rijhwani SK, Shanks JV (1998) Effects of elicitor dosage and exposure time on biosynthesis of indole alkaloids by *Catharanthus roseus* hairy root cultures. Biotechnol Prog 14: 442–449

Rischer H, Oresic M, Seppänen-Laakso T, Katajamaa M, Lammertyn F, Ardiles-Diaz W, Van Montagu MCE, Inzé D, Oksman-Caldentey KM, Goossens A (2006) Gene-to-metabolite networks for terpenoid indole alkaloid biosynthesis in *Catharanthus roseus* cells. Proc Natl Acad Sci USA 103: 5614–5619

Rudell DR, Mattheis JP (2002) Methyl jasmonate enhances anthocyanin accumulation and modifies production of phenolics and pigments in 'Fuji' apple. J Amer Soc Hort Sci 127: 435–441

Sabater-Jara AB, Almagroa L, Belchí-Navarroa S, Ferrerb MA, Barcelóa AR, Pedrenoa MA (2010) Induction of sesquiterpenes, phytoesterols and extracellular pathogenesis-related proteins in elicited cell cultures of *Capsicum annuum*. J Plant Physiol 167: 1273–1281

Saiman MZ, Mustafa NR, Schulte AE, Verpoorte R, Choi YH (2012) Induction, characterization, and NMR-based metabolic profiling of adventitious root cultures from leaf explants of *Gynura procumbens*. Plant Cell Tiss Organ Cult 109: 465–475

Saiman MZ, Mustafa NR, Pomahocova B, Verberne M, Verpoorte R, Choi YH, Schulte AE (2014) Analysis of metabolites in the terpenoid pathway of *Catharanthus roseus* cell suspensions. Plant Cell Tiss Organ Cult 117: 225–239

Saniewski M, Czapski J (1983) The effect of methyl jasmonate on lycopene and *β*-carotene accumulation in ripening red tomatoes. Cell Mol Life Sci 39: 1373–1374

Schuhr CA , Radykewicz1 T, Sagner S, Latzel C, Zenk MH, Arigoni D, Bacher A, Rohdich F, Eisenreich W (2003) Quantitative assessment of crosstalk between the two isoprenoid biosynthesis pathways in plants by NMR spectroscopy. Phytochem Rev 2: 3–16

Suh WH, Hyun SH, Kim SH, Lee SY, Choi HK (2013) Metabolic profiling and enhanced production of phytosterols by elicitation with methyl jasmonate and silver nitrate in whole plant cultures of *Lemna paucicostata*. Process Biochem 48: 1581–1586

Thabet I, Guirimand G, Guihur A, Lanoue A, Courdavault V, Papon N, Bouzid S, Giglioli-Guivarc'h N, Simkin AJ, Clastre M (2012) Characterization and subcellular localization of geranylgeranyl diphosphate synthase from *Catharanthus roseus.* Mol Biol Rep 39: 3235–3243

van der Fits L, Memelink J (2000) ORCA3, a jasmonate-responsive transcriptional regulator of plant primary and secondary metabolism. Science 289: 295–297

van der Heijden R, Jacobs DI, Snoeijer W, Hallard D, Verpoorte R (2004) The *Catharanthus* alkaloids: pharmacognosy and biotechnology. Curr Med Chem 11: 607– 628

Vázquez-Flota F, Hernández-Domínguez E, Miranda-Ham MdL, Monforte-González M (2009) A differential response to chemical elicitors in *Catharanthus roseus* in vitro cultures. Biotechnol Lett 31: 591–595

Verpoorte R, Contin A, Memelink J (2002) Biotechnology for the production of plant secondary metabolites. Phytochem Rev 1: 13–25

Wang Y, Miao Z, Tang K (2010) Molecular cloning and functional expression analysis of a new gene encoding geranylgeranyl diphosphate synthase from hazel (*Corylus avellana* L. Gasaway). Mol Biol Rep 37: 3439–3444

Westerhuis JA, van Velzen EJJ, Hoefsloot HCJ, Smilde AK (2010) Multivariate paired data analysis: multilevel PLSDA versus OPLSDA. Metabolomics 6: 119–128

Whitmer S, van der Heijden R, Verpoorte R (2002) Effect of precursor feeding on alkaloid accumulation by a strictosidine synthase over-expressing transgenic cell line S1 of *Catharanthus roseus*. Plant Cell Tiss Organ Cult 69: 85–93

Zhao J, Verpoorte R (2007) Manipulating indole alkaloid production by *Catharanthus roseus* cell cultures in bioreactors: from biochemical processing to metabolic engineering. Phytochem Rev 6: 435– 457

Chapter 4

Effect of mevalonic acid feeding on the terpenoid pathway in *Catharanthus roseus* cell suspension cultures

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Abstract

The terpenoid pathway is considered as a limiting factor in the biosynthesis of terpenoid indole alkaloid (TIA) in *Catharanthus roseus* cell suspension cultures. The limitation could be due to the competition for the isopentenyl diphosphate::dimethylallyl diphosphate utilized for different terpenoid pathways in the plastids, and includes outflow of plastidial MEP pathway precursors to other compartments and associated terpenoid pathways, both of which would result in precursor shortage to the iridoid-TIA pathway. In this study, the upstream precursor of the mevalonate pathway, i.e. mevalonic acid was fed to the *C. roseus* cell suspension cultures to evaluate the changes of metabolic flows into the different terpenoid pathways with specific attention to the distribution of C5-units into sterols (triterpenoid, C30), carotenoids (tetraterpenoid, C40), and TIAs (monoterpenoid, C10). This study showed that the sterol level was increased 72 hours after feeding mevalonic acid at a low level (0.2 mM final concentration). However, the carotenoid level was not different from the control and TIA level did not show a clear enhancement. When a higher level (3.3 mM final concentration) of mevalonic acid was fed into the cell cultures, the loganic acid level was higher than the control after 24 hours, but the TIA level was not significantly different. After 72 hours, loganic acid and TIA levels were significantly decreased and tryptamine had accumulated in the mevalonate fed cells. While carotenoids remained unaffected, sterols level was 41% increased after 24 hours and the accumulation was 111% higher than the control level after 72 hours. These results indicate that feeding mevalonic acid does not increase the levels of TIA and carotenoid in the MEP pathway but increased fluxes in the mevalonate pathway leading to sterols.

Introduction

In *Catharanthus roseus*, the terpenoid indole alkaloids (TIAs), of which some are pharmaceutically important compounds, derive from the metabolic precursors of two different biosynthetic pathways, i.e. tryptamine from the shikimate-tryptophan pathway and secologanin from the terpenoid-iridoid pathway (El-Sayed and Verpoorte 2007). Terpenoid biosynthesis in plants occurs via two distinct metabolic routes, the mevalonate (MVA) pathway which is localized in the cytosol, and the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway which is present in the plastids (Rohmer 1999). Both MVA and MEP pathway produce the terpenoid building blocks: isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), but each pathway leads to a distinct set of terpenoid derivatives (**Fig. 1**).

The iridoids in *C. roseus* are synthesized from geranyl diphosphate (GPP) supplied by the MEP pathway (Contin et al. 1998). Precursor feeding studies in *C. roseus* cell suspension cultures showed that the terpenoid pathway leading to the iridoids is the rate-limiting step for TIA biosynthesis (Moreno et al. 1993; Whitmer et al. 2002). This could be due to the lack in the biosynthesis of precursors or intermediates associated to low gene expression or enzyme activity in the MEP pathway. In addition, it might be due to the competition at the level of IPP::DMAPP and GPP with conversion steps favoring either geraniol and derived monoterpenoids and iridoids, versus geranylgeranyl diphosphate (GGPP) and derived carotenoids (Saiman et al. 2014).

There is evidence of interaction between the IPP::DMAPP pools in the cytosolic mevalonate and plastidial MEP pathways (Arigoni et al. 1997; Schuhr et al. 2003; Hemmerlin et al. 2003; Laule et al. 2003). Laule et al. (2003) showed an existence of cross-talk or interaction between the cytosolic mevalonate and the plastidial MEP pathway by studying the levels of gene transcriptions and several metabolites (sterols, carotenoids, chlorophylls) after adding specific inhibitors of the respective pathways in *Arabidopsis thaliana* seedlings. Their results show that sharing of isopentenyl precursors between both pathways may occur but only in a unidirectional process, from plastid to cytosol, i.e. the MEP pathway may feed intermediates to the cytosolic mevalonate pathway and not vice versa. This redirection of carbon resources could indicate that IPP::DMAPP from the MEP pathway are leaked to the cytosolic terpenoid pathway, when there is an increased demand but a limiting supply of the IPP::DMAPP C5-units from the mevalonate pathway. Under such circumstances, the cytosolic terpenoid pathways could be competing for the same intermediates as the carotenoid and the iridoid-TIA pathway, which could result in limitations of precursor supply to the iridoid-TIA pathway. Therefore, supplementing the *C. roseus* cell cultures with mevalonic acid might prevent the outflow of MEP pathway intermediates and improve C5 availability for carotenoids and monoterpenoids biosynthesis.

The objective of this study was to evaluate the effect of mevalonic acid feeding on *C. roseus* cell metabolism with specific attention to the distribution of C5-units into representative terpenoid groups: sterols (triterpenoid, C30), carotenoids (tetraterpenoid, C40), and TIAs (monoterpenoid, C10). In this study, we want to see if adding mevalonic acid could change the distribution of C5 precursor in terpenoid groups and if there is indirect evidence of exchange of C5 precursors.

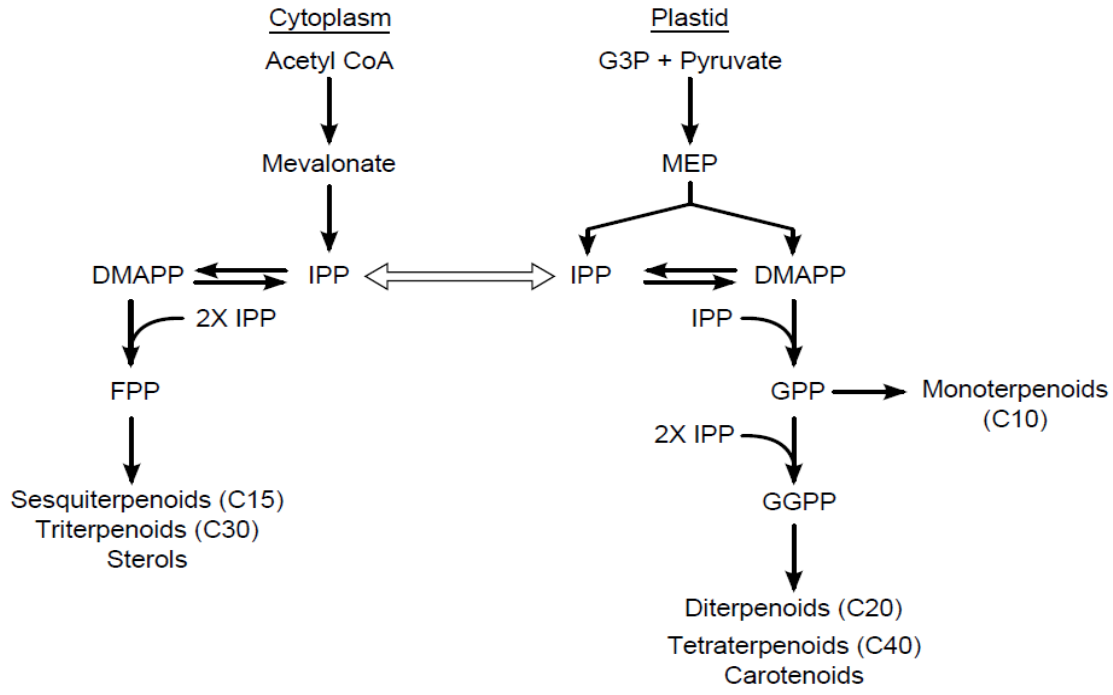

Fig. 1 Mevalonate pathway and 2-*C*-methyl-D-erythritol 4-phosphate pathway leading to different terpenoid groups. IPP: isopentenyl diphosphate, DMAPP: dimethylallyl diphosphate, GPP: geranyl diphosphate, FPP: farnesyl diphosphate, GGPP: geranylgeranyl diphosphate.

Materials and Methods

Cell culture materials

Cell suspension cultures of *Catharanthus roseus* (cell-line CRPP) were used in this study and the cultivation conditions are described in **Chapter 3**. For the experiment, 20 ml (ca. 4 g fresh weight) of two-week-old cell suspension cultures were inoculated into 50 ml fresh culture medium and maintained under the standard cultivation conditions for 4 days prior to the feeding experiment.

Mevalonic acid preparation

Mevalonic acid was prepared by addition of 130 mg of mevalonolactone (Sigma-Aldrich, St. Louis, MO, USA) to 5.5 ml of 0.2 N KOH. The solution was heated at 50 ^{o}C for 15 min. After cooling down to room temperature, the solution was adjusted to pH 7.3 with 0.1 N HCl. Water was added to the solution to achieve the total volume of 10 ml (Popjak 1969). The solution was sterilized through a 0.22 µm Millex^{TM} filter (Millipore, Bedford, MA, USA). Control solution was prepared with the same solvent without mevalonic acid.

Mevalonate feeding and metabolite analysis

Mevalonic acid solution was fed into the 4-day-old cell suspension cultures to achieve final concentrations of 0.2 mM (low mevalonate feeding) and 3.3 mM (high mevalonate feeding). The corresponding control cultures received the same amounts of solution without mevalonic acid. At selected time-points flasks were harvested in triplicate for control and treated samples; regarding the low-dosage treatment flasks were harvested at 0, 2, 6, 24, 48, and 72 hours after feeding, and for the high-dosage treatment at 24 and 72 hours after feeding. The cells were filtered under reduced pressure, subsequently washed three times with deionized water, and lyophilized for 72 hours. Terpenoid indole alkaloids, carotenoids and phytosteroids were analyzed using high performance liquid chromatography-diode array detector (HPLC-DAD) or gas chromatography-flame ionization detector (GC-FID). For the low-dosage experiment, samples were analyzed from duplicate flasks and triplicate flasks for the high-dosage treatment. In addition, samples of the low-dosage experiment (triplicate flasks) were analyzed by ^{1}H-NMR for evaluation of metabolomic changes. The analytical methods performed in this study are described in **Chapter 3**.

Statistical analysis

A *t*-test was performed on IBM SPSS Statistics 20 (SPSS Inc., Chicago, IL, USA) to determine statistical differences ($P < 0.05$) for the data from triplicate samples.

Results and Discussion

Metabolite analysis

The *Catharanthus roseus* cell suspension cultures (CRPP cell line) were fed with a low and a high level of mevalonic acid solution to achieve a final concentration of 0.2 and 3.3 mM, respectively. The lower concentration was chosen based on the previous work of Contin (1999) who fed the same concentration of loganic acid into *C. roseus* A11 cell line. The higher concentration (3.3 mM mevalonic acid) was previously applied by Moreno et al. (1993) to the *C. roseus* A12A2 cell line, in which at that level the production of C30 (steroid) biosynthetic pathway was found to be saturated in *Nicotiana tabacum* suspension cultures (Threlfall and Whitehead 1988).

Figure 2 and **3** show sterol levels in *C. roseus* cell suspension cultures at low and high mevalonic acid concentrations. For the low mevalonic acid feeding, there were no differences in both treated and control samples after 24 hours, but after 72 hours the levels of campesterol, *β*-sitosterol, and stigmasterol were 38%, 45%, and 24% increased, respectively,

in the mevalonate fed-cells compared to the controls. Analysis of the sterol accumulation upon high mevalonic acid feeding shows that after 24 hours campesterol and *β*-sitosterol were 49% and 31% increased, respectively ($P < 0.05$). After 72 hours, the mevalonic acid fed-cells accumulated 126% higher campesterol and 51% higher *β*-sitosterol levels than the controls ($P < 0.05$). No significant increase in stigmasterol level was observed after 24 hours of high mevalonic acid feeding, whereas 152% increased level was observed 72 hours after the treatment. These results indicate that the exogenously added mevalonic acid affects the sterol levels and distribution in a selective dose and time dependent way in the *C. roseus* cell cultures.

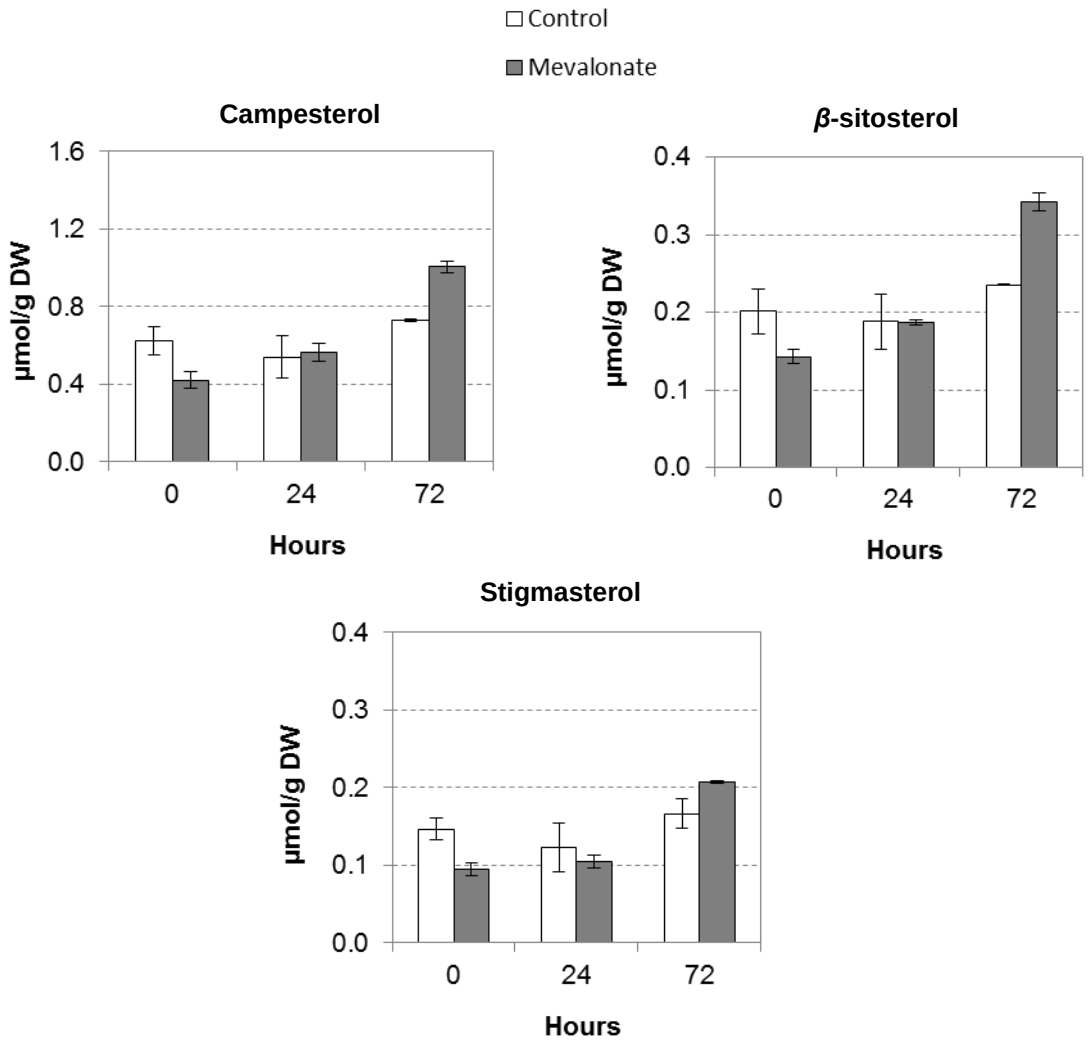

Fig. 2 Sterol production in CRPP cell line fed with a low concentration of mevalonic acid (0.2 mM) or controls measured by GC-Flame Ionization Detector (FID). Results are the mean of two replicates; error bars indicate the two values.

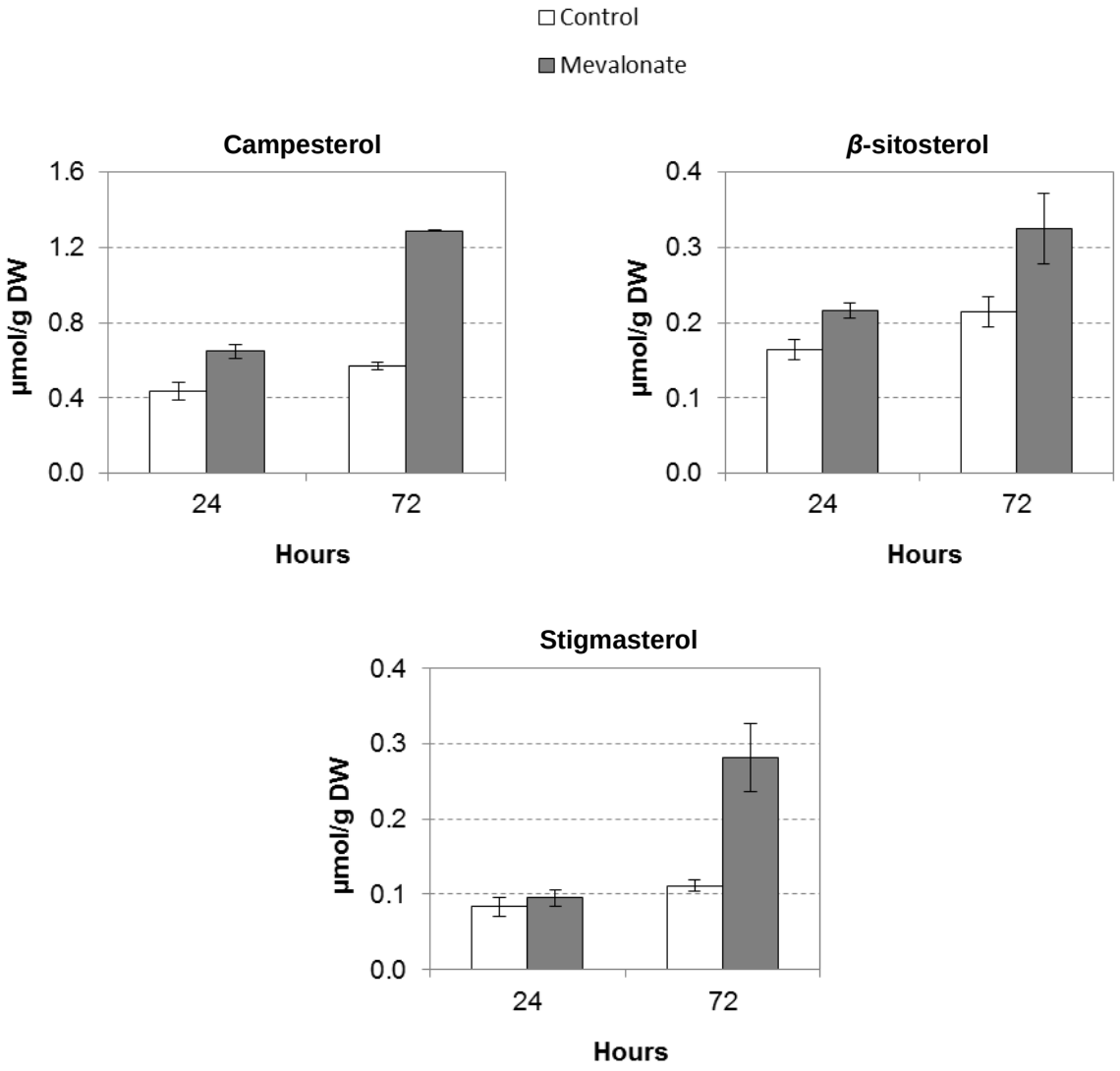

Fig. 3 Sterol production in CRPP cell line fed with a high concentration of mevalonic acid (3.3 mM) or controls measured by GC-Flame Ionization Detector (FID). Results are the mean of three replicates; error bars indicate standard deviation of the mean.

The addition of low or high mevalonic acid feeding did not seem to have any effect on the carotenoid levels compared to their controls (**Fig. 4** and **5**). It is thus presumed that the addition of exogenous mevalonic acid has no or little influence on the flux toward GGPP leading to the carotenoids production under the conditions tested.

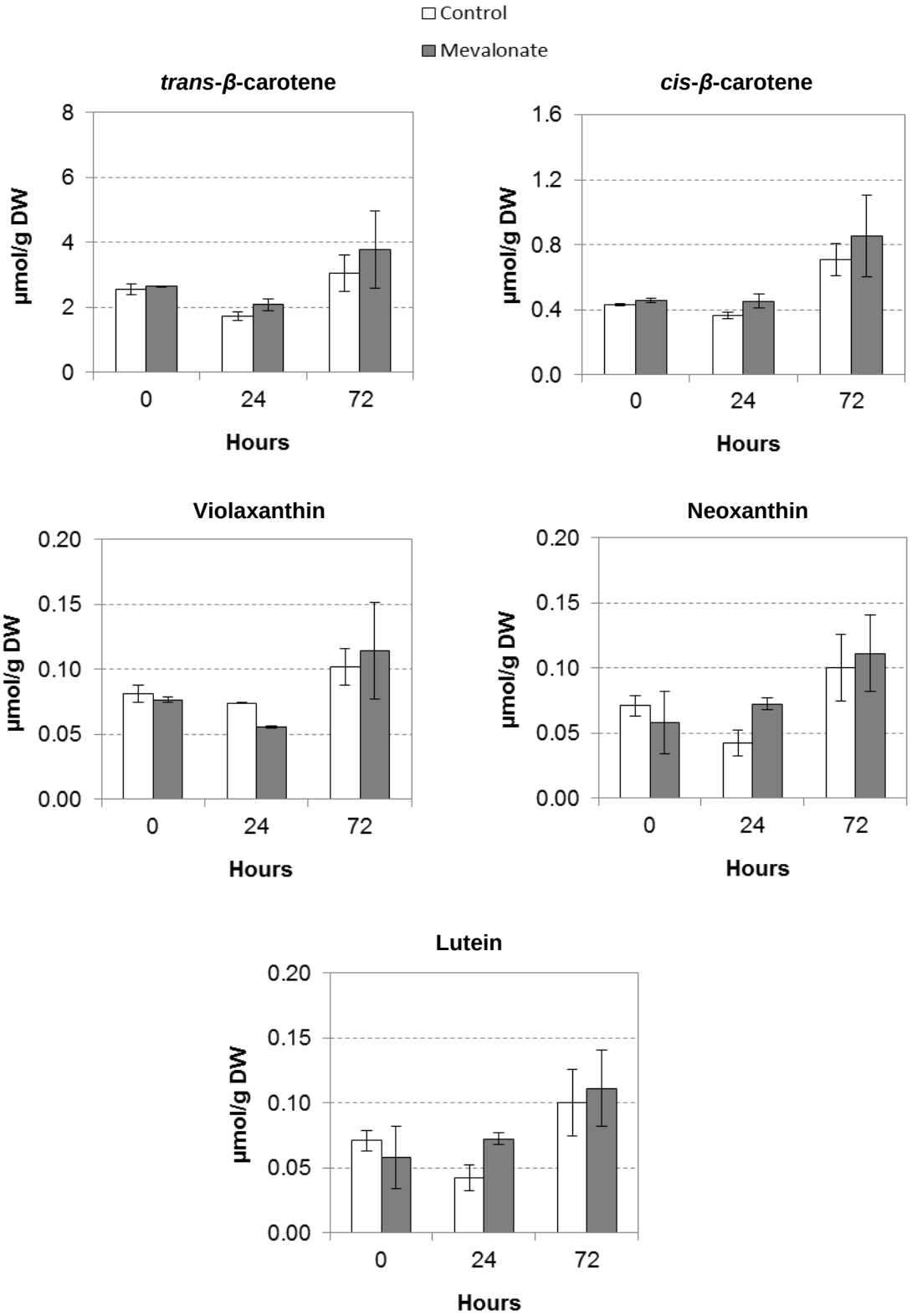

Fig. 4 Carotenoid production in CRPP cell line fed with a low concentration of mevalonic acid (0.2 mM) or controls measured by HPLC-Diode Array Detector (DAD). Results are the mean of two replicates; error bars indicate the two values.

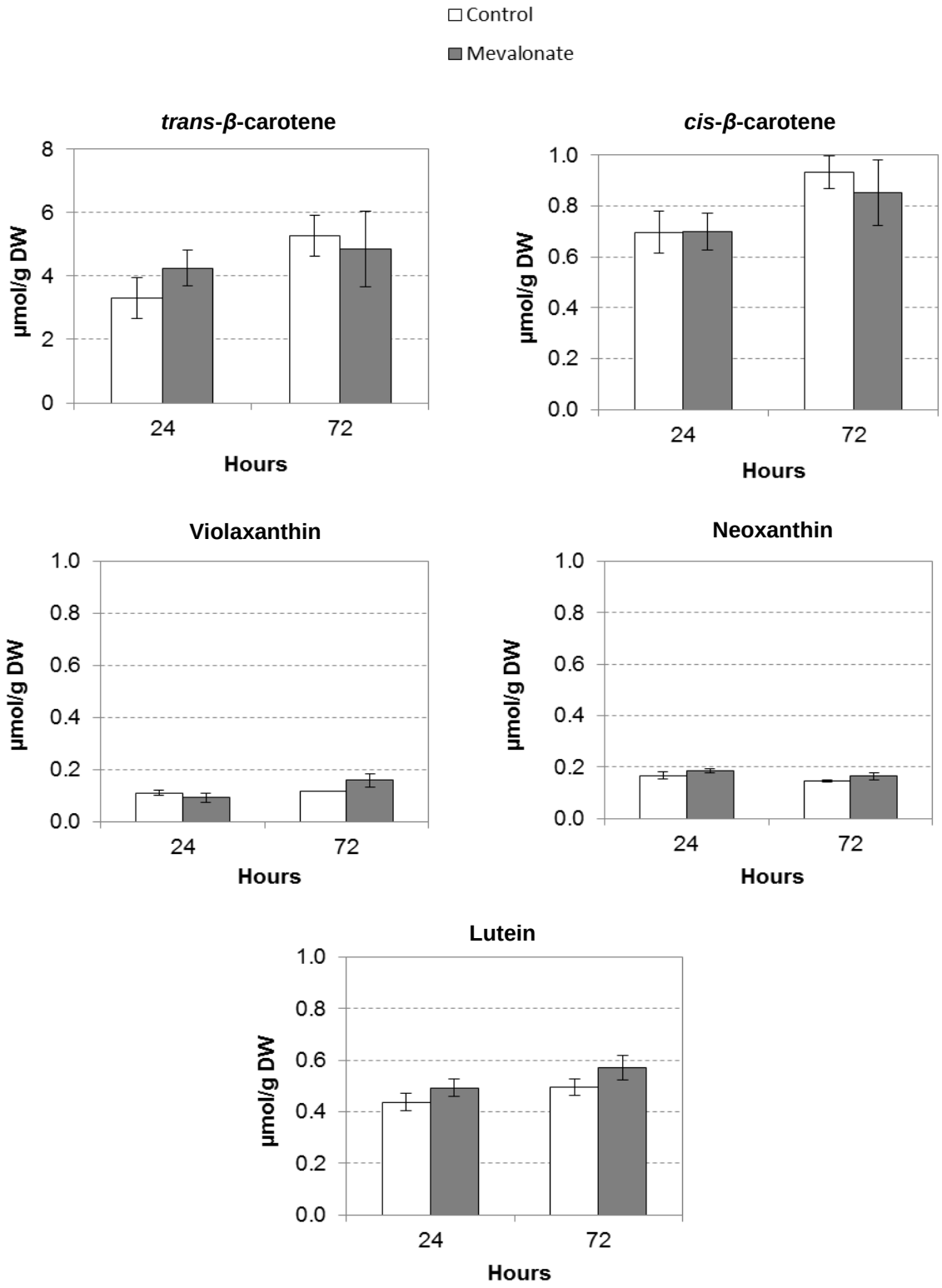

Fig. 5 Carotenoid production in CRPP cell line fed with a high concentration of mevalonic acid (3.3 mM) or controls measured by HPLC-Diode Array Detector (DAD). Results are the mean of three replicates; error bars indicate standard deviation of the mean.

Nevertheless, we noted that both control and treated cultures in the high-mevalonate feeding had a higher carotenoid content than in the low-dosage treatment regarding all individual components. This might be due to a solvent effect or biological variation throughout the cultivation of the cell line. In addition, we observed a pronounced change in the intensity of the green color in the high-mevalonate feeding experiment, as the treated cells turned from green to dark green after 72 hours (**Fig. 6**), which was not observed at low-dosage feeding. Analysis on the chlorophyll levels in the low or high mevalonate feeding did not show any differences between control and the treated cultures (**Fig. 7**). Therefore, the mevalonate-induced color change is neither associated to a difference in carotenoid nor to chlorophyll levels, which leaves the possibility that adjuvants of chlorophylls or carotenoids are affected by mevalonate-derived products and subsequently intensify the color, or that other color components are increased.

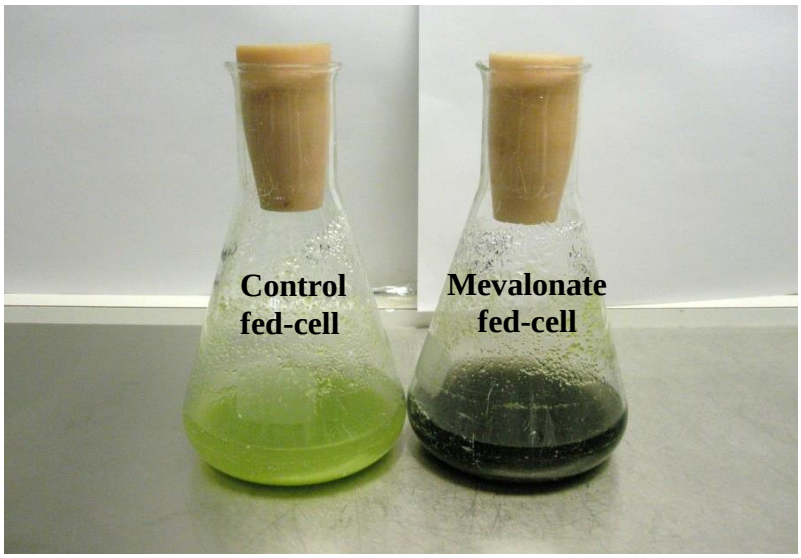

Fig. 6 Comparison of the control and high mevalonic acid fed-cell (3.3 mM) cultures after 72 hours.

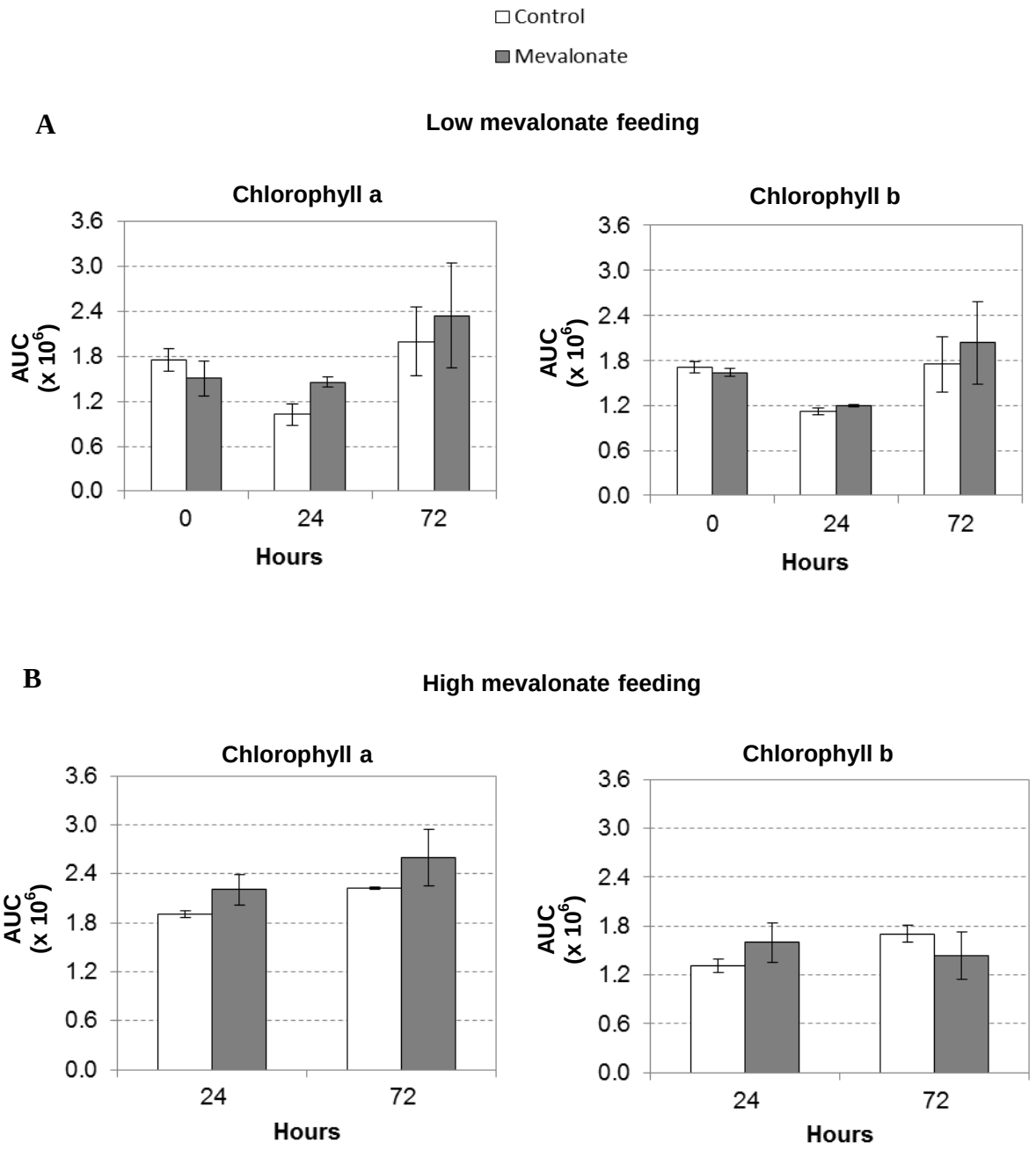

Fig. 7 Chlorophyll levels in CRPP cell line fed with A) low (0.2 mM) or B) high (3.3 mM) mevalonic acid concentration and their respective controls as measured by HPLC-Diode Array Detector (DAD). Results for low mevalonic acid feeding are the mean of two replicates; error bars indicate the two values. Results for high mevalonic acid feeding are the mean of three replicates; error bars indicate standard deviation of the mean. AUC is area under the curve value.

The cell culture used in this study is a TIA-producing *C. roseus* cell line accumulating among others: strictosidine, serpentine, tabersonine, and loganic acid, the iridoid alkaloid precursor from the terpenoid pathway. **Figure 8** shows that the loganic acid production seems to be increased by the low mevalonic acid feeding after 72 hours (twofold higher level; 1.16 µmol/g DW), however, the result is not statistically significant due to insufficient sample replicates. The levels of strictosidine, serpentine, and tabersonine were found to be higher in the low mevalonic acid fed-cells compared to the control after 72 hours. However, the levels of strictosidine and serpentine were about the same as at 0 hour, whereas the tabersonine level was lower than at 0 hour. Therefore, the conclusion is that there is no real enhancement in the TIA production after feeding 0.2 mM mevalonic acid to the *C. roseus* cell suspension culture. But it seems that in the controls, TIA levels become lower during time, whereas in the fed cultures it seems to go up. Previous studies by Krueger and Carew (1978), and Moreno et al. (1993) showed no effect on TIA levels in *C. roseus* cell suspension cultures after feeding 0.76 mM and 3.3 mM mevalonic acid, respectively. A similar result was also found by Morgan and Shanks (2000) who fed 52 µM – 104 µM of mevalonic acid to the 17 – 21 days old *C. roseus* hairy root cultures.

A different result was found for loganic acid and TIA levels at the higher concentration of mevalonic acid (3.3 mM) (**Fig. 9**). A twofold increase of loganic acid was observed after 24 hours of mevalonic acid treatment, however TIA levels, i.e. strictosidine, serpentine, and tabersonine were not significantly different compared to the control at that time-point ($P < 0.05$). Subsequently, the levels of loganic acid, strictosidine, and tabersonine dropped below the control level ($P < 0.05$), while tryptamine accumulated (3.5 µmol/g DW) in the mevalonate fed-cells after 72 hours (**Fig. 10**). In addition, no loganin and secologanin accumulated in the treated cells. As alkaloid levels are not increased if compared to the controls, the accumulation of tryptamine in the cell is probably due to the lower supply of secologanin to form strictosidine associated to the decreased level of loganic acid after 72 hours treatment. This indicates an adverse/negative effect of the abundant supply of mevalonic acid on loganic acid production. Interestingly, serpentine level was not affected which could be due to the fact that the anhydronium alkaloid serpentine is too polar to be excreted from the vacuoles, and thus will not be catabolized. The results in this study are different from those reported by Moreno et al. (1993) who found insignificant changes of strictosidine and ajmalicine levels at 72 and 120 hours after feeding 3.3 mM of mevalonic acid. The contradictory results could be due to the different cell lines employed in both studies. Saiman et al. (2014) showed that accumulation of terpenoid groups in *C. roseus* cell

suspensions can vary greatly dependent on the specific cell line, which is related to a pronounced difference in the expression of most relevant associated pathway genes in the cell lines.

Courdavault et al. (2005a, 2005b) showed that protein prenylation is involved in the induction of the expression of some of the early stage of monoterpenoid biosynthetic pathway genes (i.e. *DXS*, *DXR*, *G10H* [or *G8O*]), while it has no effect on *SLS*, *TDC*, and *STR* transcript levels. As this prenylation is connected to the mevalonate pathway, it would be interesting to further study the effect of mevalonate feeding on the induction of the activity of these enzymes in the cell culture.

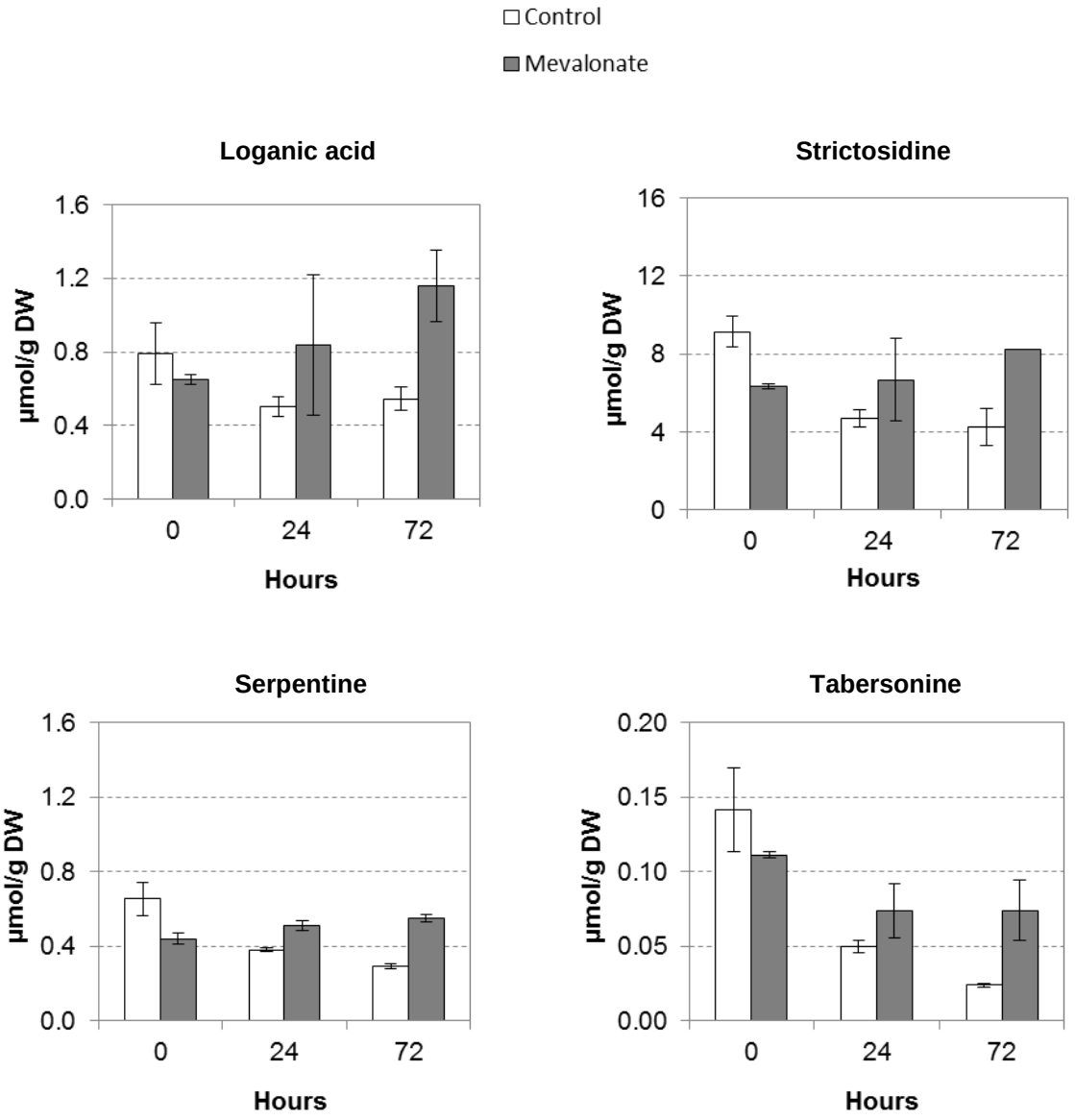

Fig. 8 Loganic acid and terpenoid indole alkaloid production in CRPP cell line fed with a low concentration of mevalonic acid (0.2 mM) or controls measured by HPLC-Diode Array Detector (DAD). Results are the mean of two replicates; error bars indicate the two values.

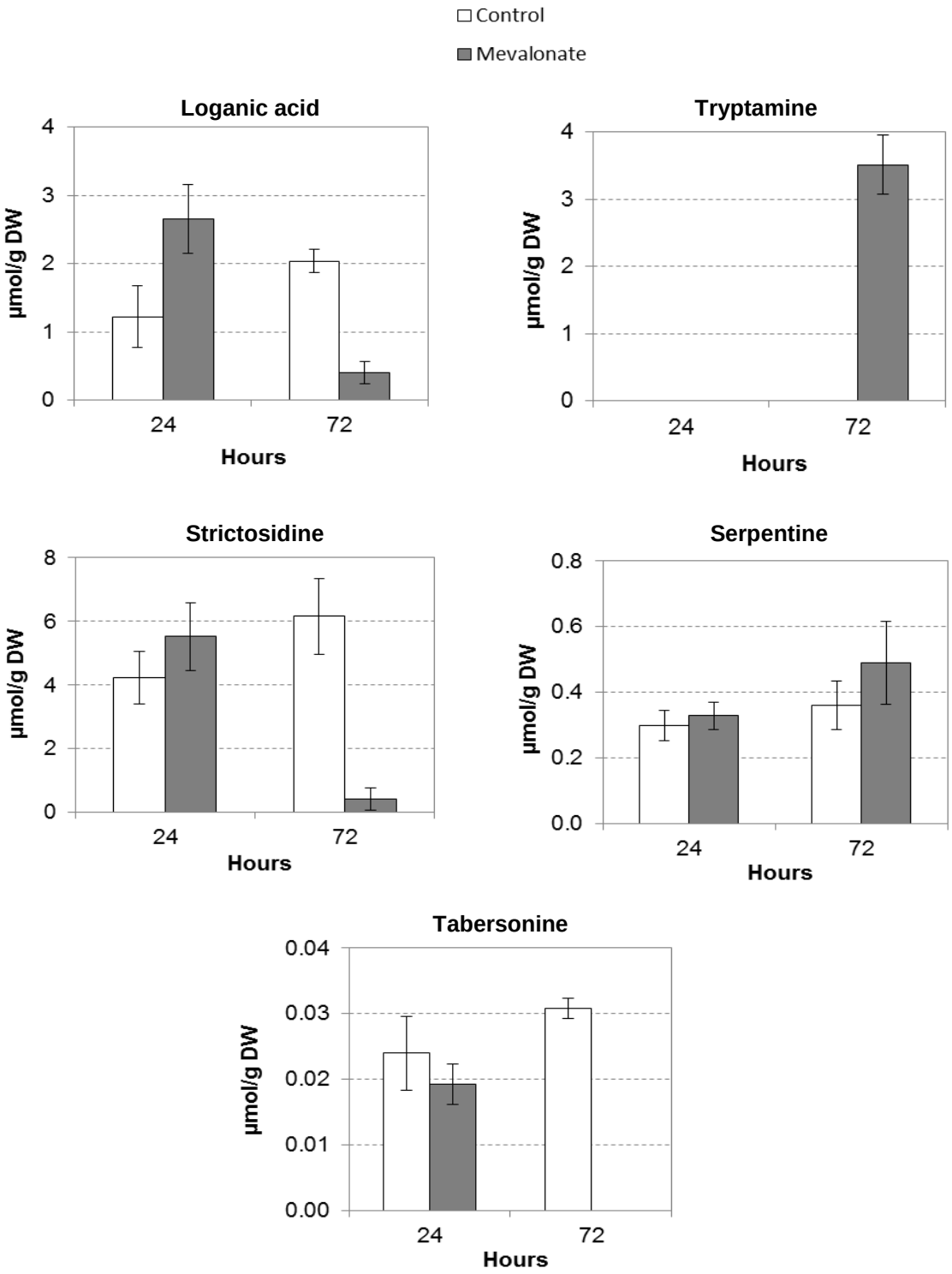

Fig. 9 Loganic acid and terpenoid indole alkaloid production in CRPP cell line fed with a high concentration of mevalonic acid (3.3 mM) or controls measured by HPLC-Diode Array Detector (DAD). Results are the mean of three replicates; error bars indicate standard deviation of the mean.

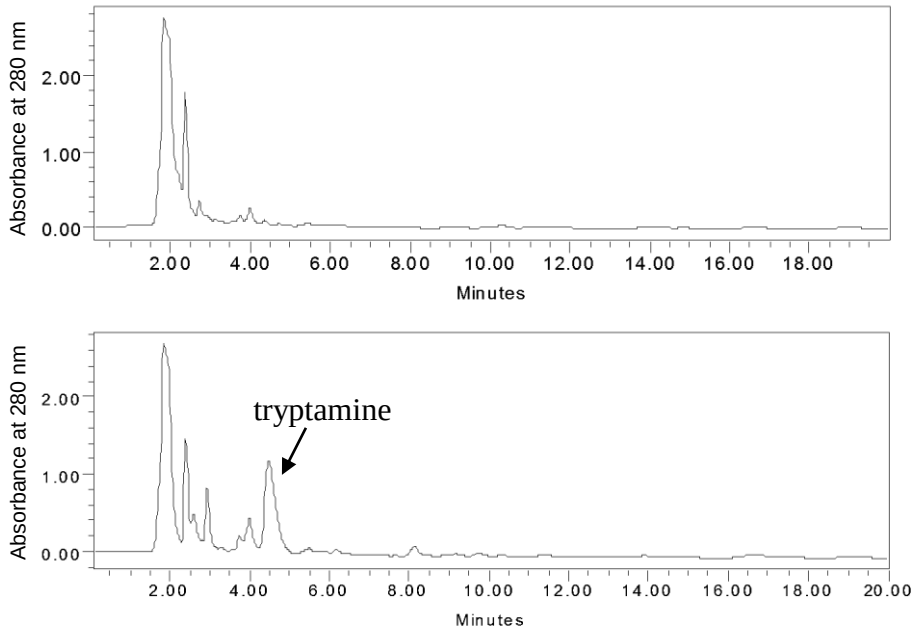

Fig. 10 Chromatograms of high performance liquid chromatography-diode array detector (HPLC-DAD) of control (top) and high mevalonic acid fed-cells (bottom) measured at 280 nm UV wavelength. Tryptamine signal was detected in the high mevalonic acid fed-cells (3.3 mM) after 72 hours.

Total accumulation and C5 distribution

The levels of sterols, carotenoids, and TIAs measured in this study and the distribution of the C5 precursors into the respective groups of metabolite are presented in **Fig. 11**. Apparently, feeding the precursor mevalonic acid to the *C. roseus* cell suspension culture resulted in an increase of sterols. Low mevalonic acid concentration (0.2 mM), resulted in 37% increase of total sterols level after 72 hours compared to the control. In the high mevalonate feeding (3.3 mM), total sterols level was already 40% higher in the treated cells at 24 hours and increased to 111% after 72 hours compared to the control ($P < 0.05$). In the *C. roseus* cell culture, campesterol was found to be the highest accumulated phytosterol, which is in accordance with the report of Suzuki et al. (1995). Feeding mevalonic acid at low concentration maintained campesterol as the major sterol. Campesterol was found as the major sterol (68%) compared to the sum of *β*-sitosterol and stigmasterol (32%) in the high mevalonic acid feeding at 24 hours after treatment and the ratio was maintained after 72 hours.

As compared to the terpenoid groups measured in this study, the C5 distribution was largest towards the carotenoid production. However, no significant difference of carotenoids level and C5 distribution was observed after mevalonic acid feeding. Consequently, there is

no indirect evidence that the added mevalonic acid reduces an outflow of MEP pathway intermediates (IPP and DMAPP) that might limit carotenoid production. There is no clear enhancement of TIA after low mevalonate feeding as the level was about the same as at 0

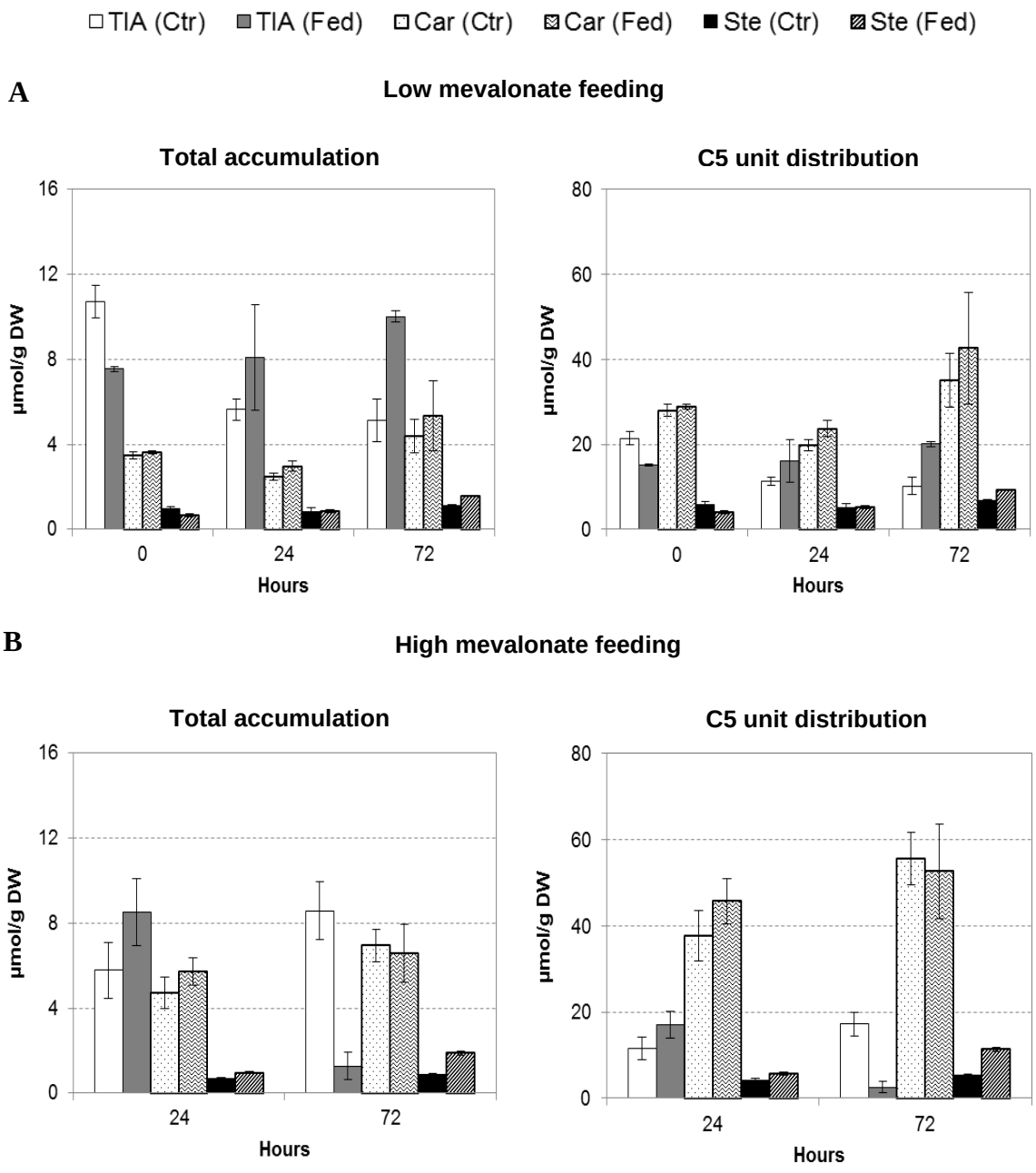

Fig. 11 Total accumulation of terpenoid indole alkaloids, carotenoids, and sterols and the C5 precursor distribution in CRPP cell line fed with A) low (0.2 mM) or B) high (3.3 mM) mevalonic acid concentration and their respective controls. Results for low mevalonic acid feeding are the mean of two replicates; error bars indicate the two values. Results for high mevalonic acid feeding are the mean of three replicates; error bars indicate standard deviation of the mean.

hour. Whereas, an increased the mevalonate concentration (3.3 mM) significantly decreased the TIA level after 72 hours ($P < 0.05$). It is clear that the added mevalonic acid precursor is distributed towards the sterol pathway.

^{1}H-NMR and multivariate data analysis

Samples of low mevalonate feeding at 0, 2, 6, 24, 48, and 72 hours after treatment were subjected to ^{1}H-NMR analysis and multivariate data analysis. The unsupervised clustering method known as principal component analysis (PCA) was performed. **Figure 12** shows the PCA score plot of this study using Pareto method as the scaling technique. The samples were separated based on the cell age: 0 – 24 hours samples were separated on the positive side of PC1, while 48 – 72 hours samples were separated on the negative side of PC1. However, no clear separation between the mevalonate fed cells and their controls at different time points is shown in the PCA. This indicates that the differences due to cell age are larger than due to the feeding. Therefore, another technique called partial least squares-discriminant analysis (PLS-DA) was applied to the same bucketed ^{1}H-NMR spectra. In contrast to PCA which projected the maximum variation within all the samples, PLS-DA is a supervised multivariate data analysis which searches for the differences between defined classes; in this case mevalonate fed cells and controls. As for PCA, the PLS-DA did not exhibit a valid model that could distinguish between the mevalonate feeding and the control samples. In addition, a PLS-DA analysis using only 48 – 72 hours samples did not show separation between the treated and control samples either. This indicates that the profiles of metabolites extracted with methanol-phosphate buffer solvent employed for NMR sample preparation do not significantly differ for the low mevalonate treated-cells and the control samples, and thus that the low mevalonate feeding (0.2 mM) does not have a major effect on the metabolism of the cells. The high mevalonate treated-cells (3.3 mM) will be analyzed to evaluate further metabolic changes upon a high mevalonic acid feeding.

Conclusion

The results obtained in this study show that feeding either a low or a high concentration of mevalonic acid to the TIA-accumulating *C. roseus* cell suspension culture (CRPP line) do not increase the absolute levels of TIA or carotenoids in the MEP pathway. Moreover, the TIA levels at high mevalonate feeding are strongly repressed at a point up-stream of loganic acid. The production of sterols derived from the mevalonate pathway is significantly increased after addition of the mevalonic acid in a dose dependent way.

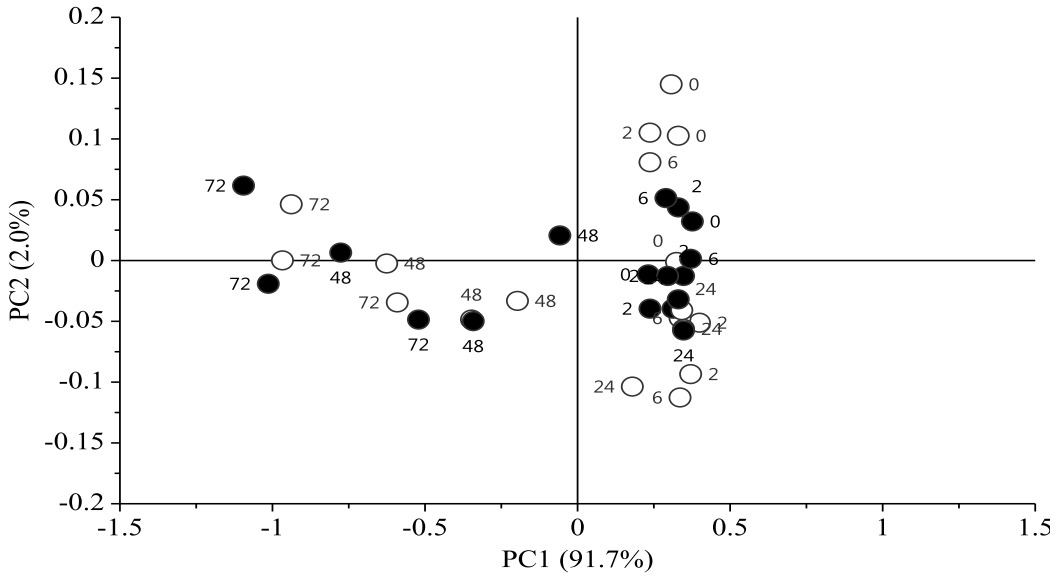

Fig. 12 Score plot of principal component analysis (PCA) of low mevalonic acid fed-cell (●) and control (○) samples of the CRPP cell line measured by ^{1}H-NMR. The numbers in the score plot are harvesting time (hour) after treatments.

Acknowledgements

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References

Arigoni D, Sagner S, Latzel C, Eisenreich W, Bacher A & Zenk MH (1997) Terpenoid biosynthesis from 1-deoxy-D-xylulose in higher plants by intramolecular skeletal rearrangement. Proc Natl Acad Sci USA 94: 10600–10605

Courdavault V, Burlat V, St-Pierre B, Giglioli-Guivarc'h N (2005a) Characterisation of CaaX-prenyltransferases in *Catharanthus roseus*: relationships with the expression of genes involved in the early stages of monoterpenoid biosynthetic pathway. Plant Sci 168: 1097–1107

Courdavault V, Thiersault M, Courtois M, Gantet P, Oudin A, Doireau P, St-Pierre B, Giglioli-Guivarc'h N (2005b) CaaX-prenyltransferases are essential for expression of genes involved in the early stages of monoterpenoid biosynthetic pathway in *Catharanthus roseus* cells. Plant Mol Biol 57: 855–870

Contin A, van der Heijden R, Lefeber AWM, Verpoorte R (1998) The iridoid glucoside secologanin is derived from the novel triose phosphate/pyruvate pathway in a *Catharanthus roseus* cell culture. FEBS Letters 434: 413–416

Contin A (1999) The biosynthesis of secologanin in *Catharanthus roseus* cell suspension cultures. PhD Thesis, Leiden University, The Netherlands. Chapter 7, Loganic acid methyltransferase activity in *Catharanthus roseus* cell cultures, pp. 59–67. ISBN 9074538436

El-Sayed M, Verpoorte R (2007) *Catharanthus* terpenoid indole alkaloids: biosynthesis and regulation. Phytochem Rev 6: 277–305

Hemmerlin A, Hoeffler JH, Meyer O, Tritsch D, Kagan IA, Grosdemange-Billiard C, Rohmer M, Bach TJ (2003) Cross-talk between the cytosolic mevalonate and the plastidial methylerythritol phosphate pathways in Tobacco Bright Yellow-2 cells. J Biol Chem 18: 26666-26676

Krueger RJ, Carew DP (1978) *Catharanthus roseus* tissue culture: the effects of precursors on growth and alkaloid production. J Nat Prod 41: 327–331

Laule O, Fürholz A, Chang H-S, Zhu T, Wang X, Heifetz PB, Gruissem W, Lange BM (2003) Crosstalk between cytosolic and plastidial pathways of isoprenoid biosynthesis in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 100: 6866–6871

Moreno PRH, van der Heijden R, Verpoorte R (1993) Effect of terpenoid precursor feeding and elicitation on formation of indole alkaloids in cell suspension cultures of *Catharanthus roseus*. Plant Cell Rep 12: 702–705

Morgan JA, Shanks JV (2000) Determination of metabolic rate-limitations by precursor feeding in *Catharanthus roseus* hairy root cultures. J Biotechnol 79: 137–145

Popjak G (1969) Enzymes of sterol biosynthesis in liver and intermediates of sterol biosynthesis. In: Clayton RB (ed.) Methods in Enzymology, Vol. 15, Academic Press, New York, pp. 393–454

Rohmer M (1999) The discovery of a mevalonate-independent pathway for isoprenoid biosynthesis in bacteria, algae and higher plants. Nat Prod Rep 16: 565–574

Saiman MZ, Mustafa NR, Pomahocova B, Verberne M, Verpoorte R, Choi YH, Schulte AE (2014) Analysis of metabolites in the terpenoid pathway of *Catharanthus roseus* cell suspensions. Plant Cell Tiss Organ Cult 117: 225–239

Schuhr CA , Radykewicz1 T, Sagner S, Latzel C, Zenk MH, Arigoni D, Bacher A, Rohdich F, Eisenreich W (2003) Quantitative assessment of crosstalk between the two isoprenoid biosynthesis pathways in plants by NMR spectroscopy. Phytochem Rev 2: 3–16

Suzuki H, Inoue T, Fujioka S, Saito T, Takatsuto S, Yokota T, Murofushi N, Yanagisawa T, Sakurai A (1995) Conversion of 24-methylcholesterol to 6-oxo-24-methylcholestanol, a putative intermediate of the biosynthesis of brassinosteroids, in cultured cells of *Catharanthus roseus.* Phytochemistry 40: 1391–1397

Threlfall DR, Whitehead IM (1988) Co-ordinated inhibition of squalene synthase and induction of enzymes of sesquiterpenoid phytoalexin biosynthesis in cultures of *Nicotiana tabacum*. Phytochemistry 27: 2567-2580

Whitmer S, van der Heijden R, Verpoorte R (2002) Effect of precursor feeding on alkaloid accumulation by a strictosidine synthase over-expressing transgenic cell line S1 of *Catharanthus roseus*. Plant Cell Tiss Organ Cult 69: 85–93

Chapter 5

Effect of geraniol feeding and jasmonic acid elicitation on terpenoid indole alkaloids accumulation in *Catharanthus roseus* cell suspension cultures

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Abstract

Catharanthus roseus is an important medicinal plant producing terpenoid indole alkaloids (TIA). Previous studies showed that geraniol may be limiting as an upstream precursor in the monoterpenoid pathway. Therefore, in the present study, geraniol was fed to *C. roseus* cell suspension cultures (cell-line CRPP) at increasing concentrations (0.32 – 1.62 mM). After feeding, the cell cultures were found to accumulate loganic acid (up to 10-fold) and a large new peak was detected by HPLC-DAD, in a concentration- and time-dependent manner. However, the production of strictosidine and down-stream TIA seemed unaffected upon geraniol feeding, whereas at the higher geraniol feeding concentrations (1.30 – 1.62 mM) catharanthine and tabersonine levels decreased compared to the control cultures. As determined for this cell line, JA elicitation increased strictosidine, serpentine, tabersonine, catharanthine, and loganic acid levels. The latter compound is significantly increased only at 24 hours after treating with lower JA concentration (34 and 102 μM), but did not accumulate to the level as detected by geraniol feeding. A combination treatment of geraniol feeding (1.62 mM) at day 4 and JA elicitation (102 μM) at day 5 showed a significant increased level of strictosidine (68%) compared to the single JA treatment at 72 hours after elicitation. However, the combination treatment did not further improve serpentine production while catharanthine and tabersonine levels were lower like in case of high geraniol feeding. These results show that feeding geraniol to *C. roseus* cell suspension cultures leads to accumulation of loganic acid, and in combination with JA elicitation the flux towards strictosidine is increased, but not to further down-stream TIA like serpentine, catharanthine, and tabersonine. Furthermore, an improved HPLC-DAD method showed 5 new peaks in the geraniol-fed cells, the NMR spectra of the extract showed several signals similar to the proton signals as geraniol and thus point to the presence of geraniol analogues. Nevertheless, due to the complexity of the mixture, isolation of the compounds is required for further identification.

Introduction

Catharanthus roseus is a rich source of terpenoid indole alkaloids (TIA). It is one of the most extensively studied medicinal plants and consequently served as an important model system for plant biotechnology and secondary metabolites production (van der Heijden et al. 2004). The TIA are products of condensation from two biosynthetic routes; tryptamine, derived from the amino acid tryptophan (one of the products of the shikimate pathway), and secologanin derived from geraniol (product of the monoterpenoid pathway) (van der Heijden

et al. 2004). The latter is known to be synthesized from the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway (Contin et al. 1998).

Precursor feeding studies were performed in *C. roseus* cell cultures to determine the metabolic flux limitations in the TIA biosynthesis. The addition of loganic acid, loganin, or secologanin increased TIA levels in the *C. roseus* cell cultures and thus it was suggested that the TIA production in *C. roseus* cell is limited by availability of precursors in the monoterpenoid pathway (Moreno et al. 1993; Whitmer et al. 1998, 2002). Studies were also conducted to observe the effect of feeding early precursors in the monoterpenoid pathway. A study by Krueger and Carew (1978) showed that feeding geraniol, the upstream precursor of the iridoid moiety produced from geranyl diphosphate (GPP), did not affect the TIA production in *C. roseus* cell cultures. In contrast, geraniol treatment reduced growth and TIA production in compact callus clusters cultures of *C. roseus* (Zhao et al. 2001). However, Morgan and Shanks (2000) reported that feeding geraniol increased the tabersonine level in *C. roseus* hairy root cultures and Lee-Parsons and Royce (2006) also found the increase of ajmalicine production in geraniol-fed *C. roseus* cell suspension cultures. The latter results suggested that geraniol may be the limiting factor as upstream precursor of the monoterpenoid pathway.

In this study, the transient effect of geraniol feeding on the production of TIA and iridoid precursors in the *C. roseus* cell-line CRPP was analyzed. Furthermore, the effect of jasmonic acid in the geraniol-fed CRPP cell cultures was assessed to determine how jasmonate elicitation alters the precursor availability for TIA biosynthesis. Experiments were also conducted at increasing concentration of geraniol and jasmonic acid to determine the appropriate concentration treatment.

Materials and methods

Cell materials

Catharanthus roseus cell suspension cultures (cell-line CRPP-glucose) were grown in B5 medium (Gamborg et al. 1968) supplemented with 20 g/l glucose and 1.86 mg/l 1-naphthaleneacetic acid (NAA). The cell suspension cultures were placed on a gyratory shaker (110 rpm) under continuous light (10.8 – 27 µE/m^2/sec) at 25 ± 1 ^oC. To obtain sufficient biomass for the experiment, the CRPP cell line was subcultured every two weeks by subculturing 1:1 dilution of 100 ml culture into 100 ml fresh medium and equally divided to two flasks. For inoculation, two to three flasks of the two-week-old CRPP cell cultures were

randomly mixed together in one flask and subsequently 20 ml of the cell suspensions (approx. 4 g FW cells) were pipetted into 50 ml of fresh medium.

Preparation of geraniol and jasmonic acid solutions

Geraniol (Sigma-Aldrich, St. Louis, MO, USA) was diluted in 50% v/v ethanol to make a 100 mg/ml stock solution. The stock solution of jasmonic acid (10 mg/ml) was prepared by dissolving jasmonic acid (Sigma-Aldrich) in 2 ml ethanol and diluted with sterile water to acquire the total volume of 10 ml. The stock solutions were filter-sterilized using 0.22 µm Millex^{TM} filters (Millipore, Bedford, MA, USA).

Different concentrations of geraniol feeding and jasmonic acid elicitation

Geraniol stock solution was added to the cultures on the fourth day after inoculation to deliver end concentrations of 0.32, 0.65, 0.97, 1.30, and 1.62 mM geraniol. Jasmonic acid stock solution was added on the fifth day after inoculation to deliver final concentrations of 34, 102, 170, and 238 µM jasmonic acid. The same amount of solvent used in geraniol feeding (50% v/v ethanol) and jasmonate elicitation (20% v/v ethanol) were applied to the cell cultures as control. All treatments were done in triplicate flasks and cultures were harvested after 24 and 72 hours.

Geraniol feeding and combination of geraniol feeding and jasmonic acid elicitation

For geraniol feeding, the cell cultures on the fourth day after inoculation were fed with geraniol stock solution to achieve the final concentration of 1.62 mM. The cells were harvested at 0, 24, 48, and 96 hours after treatment. For feeding + elicitation, the cultures fed with geraniol on the fourth day were elicited with jasmonic acid on the fifth day after inoculation to the final concentration of 102 µM. The cells were harvested at 0, 24, and 72 hours after elicitation. The same amounts of solvent used in geraniol feeding and jasmonate elicitation were applied to the cell cultures as solvent control. Control blank cultures without any treatment were also collected at all harvesting time-points. Each treatment was performed in triplicate.

Harvesting

Samples were harvested using a Bűchner vacuum-filtration unit and rinsed with deionized water. Subsequently, the cells were immediately frozen in liquid nitrogen and

lyophilized for 72 hours to deliver biomass accumulation in dry weight (DW) per flask. Samples were kept at room temperature in the dark until further analysis.

TIA and precursors analysis

Terpenoid indole alkaloids (strictosidine, ajmalicine, serpentine, catharanthine, tabersonine, vindoline, vinblastine, vincristine) and precursors (loganic acid, loganin, secologanin, tryptophan, tryptamine) were extracted and analyzed by high performance liquid chromatography-diode array detector (HPLC-DAD) methods as described by Saiman et al. (2014). The HPLC-DAD methods were run on an Agilent Technologies 1200 series chromatograph equipped with a diode array detector and a Zorbax Eclipse XDB-C18 column (250 mm x 4.6 mm, particle size 5 µl) (Agilent Techologies Inc., Santa Clara, CA, USA).

Separation of new precursor peaks (A – E) was carried out using a 150 x 4.6 mm Luna 5 micron C18 (2) 100A column (Phenomenex Inc., Torrance, CA, USA) and performed on the same HPLC-DAD series (Agilent Techologies Inc.). The mobile phase consisted of acetonitrile (A) and 0.1% trifluoroacetic acid in water (B). The eluent profile (volume of solvent A/volume of solvent B) was: 0 – 5 min, isocratic elution with 10:90 (v/v); 5 – 30 min, linear gradient from 10:90 to 95:5 (v/v); 30 – 35 min, isocratic elution with 10:90 (v/v). The flow rate was 1.0 ml/min and the injection volume was 10 µl. The diode array detector was set at 240 nm with a UV spectrum scan from 190 – 390 nm.

Geraniol analysis

The dried cells (100 mg) were extracted two times with 5 ml ethyl acetate, vortexed, sonicated for 20 min, and centrifuged at 3500 rpm (4 ^{o}C) for 10 min. The supernatants were combined and concentrated to dryness under reduced pressure. The residue was dissolved in 400 µl methanol. For culture medium, 5 ml liquid medium was extracted with 5 ml ethyl acetate, vortexed, and centrifuged at 3500 rpm (4 ^{o}C) for 10 min. This procedure was repeated three times. The combined supernatants were concentrated to dryness and the residue was dissolved in 400 µl methanol. The samples were filtrated over miracloth prior to geraniol analysis.

Geraniol analysis was carried out using an Agilent 6890 series (Agilent Technologies Inc.) gas chromatograph (GC) equipped with 7863 series injector, a DB-5 capillary column (30 m x 0.25 mm i.d., film thickness of 0.25 um) (J&W Scientific Inc., Folsom, CA, USA) and coupled with a flame ionization detector (FID). Analysis conditions were: injector temperature: 230 ^{o}C, detector temperature: 250 ^{o}C, initial oven temperature: 100 ^{o}C. A

temperature gradient was applied running from 100 ^{o}C to 140 ^{o}C at 10 ^{o}C/min and then from 140 ^{o}C to 240 ^{o}C at 35 ^{o}C/min, and maintaining 240 ^{o}C for 20 min. Nitrogen was used as carrier gas (15.5 psi) at 1.2 ml/min flow rate and the injection split ratio was 1 to 50. The sample injection volume was 5 µl. Identification of the products was performed by comparison with standard compounds.

NMR analysis

NMR analysis was conducted according to the protocol of Kim et al. 2010. Fifty milligrams of freeze-dried cells were extracted with 1.2 ml of methanol-d_4 and 0.3 ml of potassium dihydrogen phosphate buffer in deuterium oxide (pH 6). The mixture was vortexed for 10 sec, sonicated for 10 min, and centrifuged for 15 min (14,000 rpm). An aliquot of 0.8 ml of the supernatant was transferred into an NMR tube for measurement using a Bruker AV 600 MHz spectrometer (Bruker, Karlsruhe, Germany) with cryoprobe. The ^{1}H-NMR spectra were recorded at 25 ^{o}C, consisted of 128 scans requiring 10 min and 26 sec acquisition time with following parameters: 0.16 Hz/point, pulse width of 30 (11.3 µs), and relaxation delay of 1.5 sec. Methanol-d_4 was used as the internal lock. A presaturation sequence was used to suppress the residual water signal with low power selective irradiation at the water frequency during the recycle delay. Free induction decay was Fourier transformed with a line-broadening (LB) factor of 0.3 Hz. The resulting spectra were manually phased, baseline corrected, and calibrated to trimethylsilyl propionic acid sodium salt (TMSP-d_4) at 0.0 ppm by using XWIN NMR version 3.5 (Bruker).

Two-dimensional *J*-resolved NMR spectra were acquired using 8 scans per 64 increments for F1 and 1,638.4 k for F2 using spectral widths of 6,009.6 Hz in F2 (chemical shift axis) and 50 Hz in F1 (spin–spin coupling constant axis). A 1.5 sec relaxation delay was employed. Datasets were zero filled to 512 points in F1 and both dimensions were multiplied by sine-bell functions (SSB = 0) prior to double complex FT. The *J*-resolved spectra were tilted by 45^{o}, symmetrized about F1, and calibrated to TMSP- d_4 using XWIN NMR version 3.5 (Bruker). The ^{1}H–^{1}H correlated spectroscopy (COSY) spectra were acquired with a 1.0 sec relaxation delay and 6,009.6 Hz spectral widths in both dimensions. The window function for the COSY spectra was sine-bell (SSB = 0). The HMBC spectra were obtained with 1.0 sec relaxation delay, 30183 Hz spectral width in F2 and 27164 Hz in F1. Qsine (SSB = 2.0) was used for the window function of the HMBC.

Statistical Analysis

A *t*-test was performed on IBM SPSS Statistics 20 (SPSS Inc., Chicago, IL, USA) to determine statistical differences between samples ($P < 0.05$).

Results and discussion

Evaluating different concentrations of geraniol feeding and jasmonic acid elicitation

The effects of various concentrations of geraniol and jasmonic acid in *Catharanthus roseus* cell culture (cell-line CRPP) were studied by analyzing the cell dry weight and terpenoid indole alkaloids (TIA) production including iridoid precursor accumulation. Two separate studies were conducted by feeding various end concentrations of geraniol (0.32 – 1.62 mM) and jasmonic acid (34 – 238 µM) to the cell cultures. Feeding at the final concentration of 0.32 – 1.30 mM geraniol to the *C. roseus* cell cultures showed that the cell biomass yields were quite stable and comparable to the controls (**Fig. 1**). However, cultures treated at a final geraniol concentration of 1.62 mM had a slightly lower biomass than their corresponding controls. Morgan and Shanks (2000) indicated that there were toxic effects on the growth of *C. roseus* hairy roots when geraniol was fed to the cultures at 160 mg/l (1.04 mM) or higher concentrations. Furthermore, Carriere et al. (1989) reported that the cell viability of *C. roseus* and three other plant cell cultures remained higher than 80% at 24 hours after feeding 100 mg/l geraniol (0.65 mM), but decreased rapidly beyond this concentration.

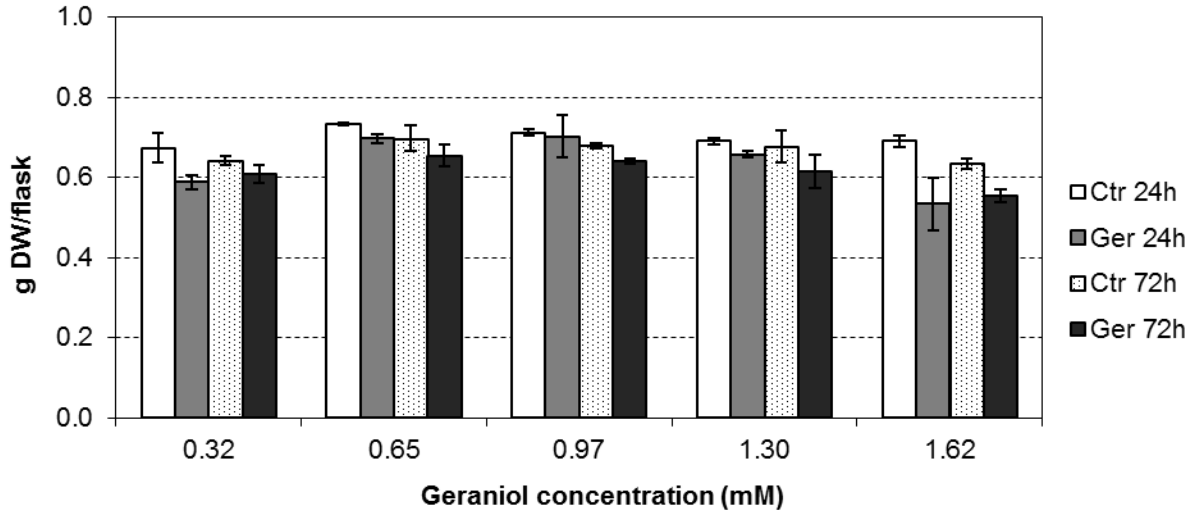

Fig. 1 Cell biomass of *Catharanthus roseus* cell culture (cell-line CRPP) treated with different concentrations of geraniol. Error bars represent standard deviations obtained from three replicates.

In the present study, the CRPP biomass accumulation (g dry weight/flask) was reduced to 23% after feeding geraniol at 1.62 mM, yet the appearance of the cultures seemed similar in density and color (green) to the solvent controls. Therefore, we concluded that it is safe to apply between 50 and 250 mg/l geraniol (0.32 – 1.62 mM) to the CRPP cell cultures without inducing strong adverse effects on biomass accumulation.

The production of TIA and TIA precursors of iridoids (i.e. loganic acid, loganin, and secologanin), tryptophan, and tryptamine was analyzed at 24 and 72 hours after feeding different concentrations of geraniol (0.32 – 1.62 mM) in the *C. roseus* cell cultures (cell-line CRPP). No tryptophan, tryptamine, loganin, and secologanin were detected in the cell cultures, only loganic acid was found (**Fig. 2**). The level of loganic acid increased in a geraniol concentration dependent manner by 2- to 10-fold at 24 hours after geraniol feeding; the maximal increase obtained was 10-fold with feeding 1.30 mM geraniol compared to control ($P < 0.05$). At 72 hours after feeding, the levels of loganic acid were consistently lower than at the 24-hour time-point, but still 2- to 3-fold higher than the corresponding controls; this loganic acid accumulation pattern suggests uptake and conversion of geraniol to loganic acid in the first 24 hours, and subsequent metabolization to other products. In addition, there is accumulation of a new peak of unknown compound observed at 240 nm at the retention time about similar to secologanin that only appeared upon feeding with geraniol. However, detailed analysis of the UV spectrum showed that the peak had a maximum UV absorption at 224 nm instead of the maximum at 238 nm for secologanin (**Fig. 3**). The level of this compound increased in a geraniol concentration dependent manner. The highest level of this unknown peak was obtained at feeding of 1.62 mM geraniol with a 2.6-fold increase at 72 hours compared to 24 hours after treatment (AUC from 4558 to 11989).

Although the level of precursors was increased after geraniol feeding, the results showed that the level of the first alkaloid strictosidine was not affected except a decrease at 24 hours after treatment with 1.62 mM geraniol, which was half of the control. Furthermore, serpentine levels were not significantly different after geraniol feeding as compared to the controls at 24 and 72 hours after treatment ($P < 0.05$). Lee-Parsons and Royce (2006) also found no significant difference in serpentine levels between control and geraniol-fed cells (0.5 mM) after 48 hours, even though ajmalicine content was slightly increased. The levels of catharanthine and tabersonine were decreased in the geraniol-fed cells at 1.30 and 1.62 mM compared to the controls ($P < 0.05$). Apparently, an increased iridoid concentration is not immediately reflected in an increased alkaloid concentration, which could be due to the lack

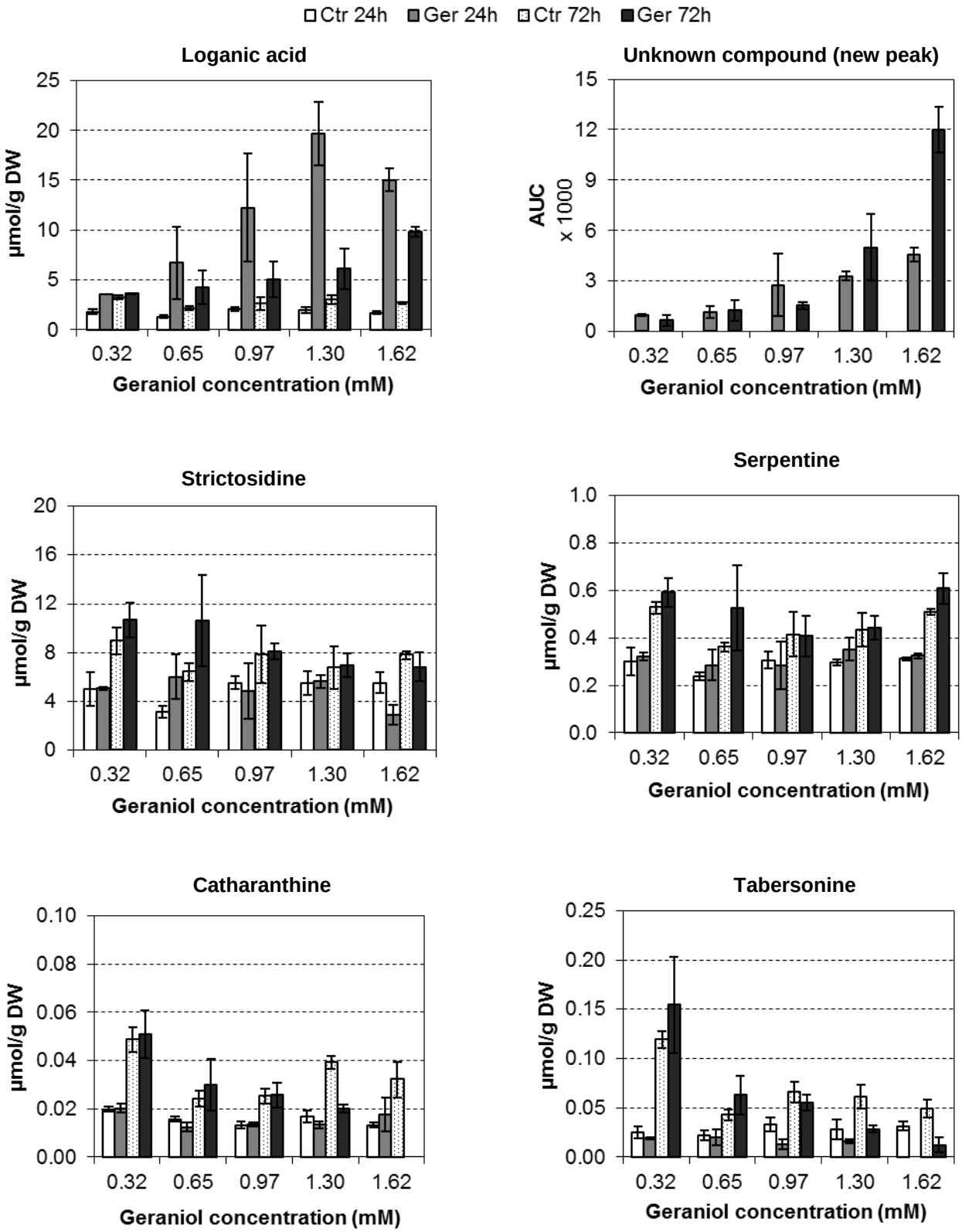

Fig. 2 Accumulation of some compounds in *Catharanthus roseus* cell culture (cell-line CRPP) treated with different concentrations of geraniol. Error bars represent standard deviations obtained from three replicates.

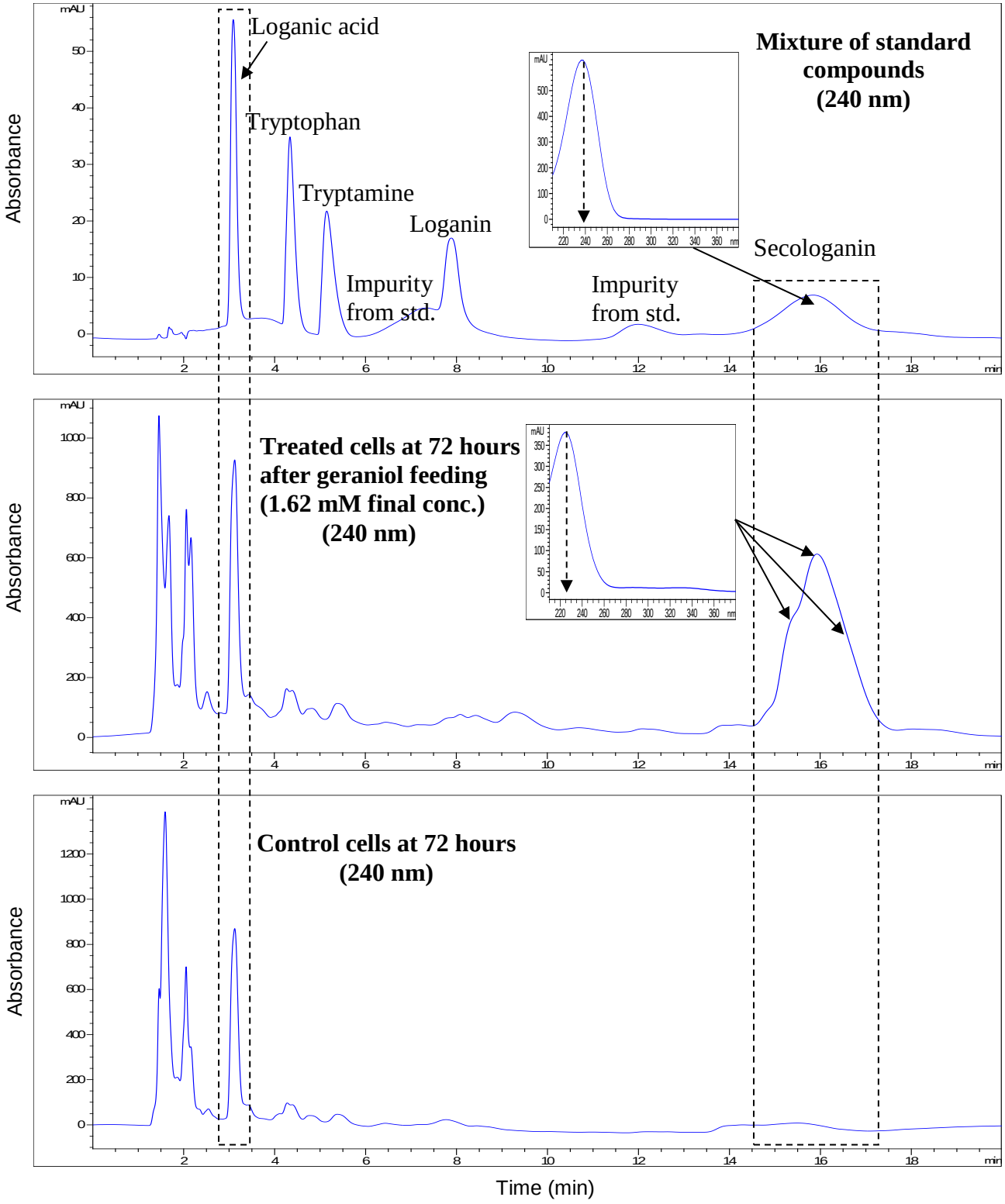

Fig. 3 Chromatograms of HPLC-Diode Array Detector (DAD) of mix standard compounds, geraniol-fed (1.62 mM) *Catharanthus roseus* cells, and control cells at 240 nm. A huge unknown peak is present in geraniol-fed cells at the retention time and UV spectrum close to secologanin.

of tryptamine. The decrease in alkaloid levels at high geraniol level might be due to a stress reaction of the cells resulting in catabolism of alkaloids.

Elicitation of the CRPP cell cultures with different JA concentrations (34 – 238 µM) showed no effect on the cell biomass yield over the tested time-period (**Fig. 4**). A study by Rijhwani and Shanks (1998) showed that final concentrations of 24 – 238 µM JA did not have a significant effect on the biomasses of *C. roseus* hairy root cultures. Therefore, it is presumed that the JA concentration range applied in this study is not toxic to the CRPP cell cultures. **Figure 5** shows the levels of loganic acid and TIA in the jasmonic acid treated-cell cultures. Jasmonic acid elicitation showed a positive effect on loganic acid accumulation but only at the lower concentration of 34 and 102 µM, at 24 hours after treatment ($P < 0.05$). In addition, it is noted that loganic acid did not accumulate to the level as produced after geraniol feeding and the new peak of the unknown compound found in the geraniol feeding experiments was not present in elicitation study (see **Fig. 2** and **3**). Regarding strictosidine, the levels were about twofold increased by JA elicitation both at 24 hours and 72 hours after treatment, and reached a maximum of 18 µmol/g DW upon elicitation. Considering serpentine, the levels were not significantly increased after 24 hours elicitation as compared to their respective controls ($P < 0.05$) except at the 102 µM elicitation treatment. At 72 hours upon elicitation, the average values of serpentine increased 25% – 62%. Although the highest serpentine level was 0.76 µmol/g DW at 72-hour time-point after feeding 102 µM JA, the variation between biological replicates was quite high and therefore the levels is not

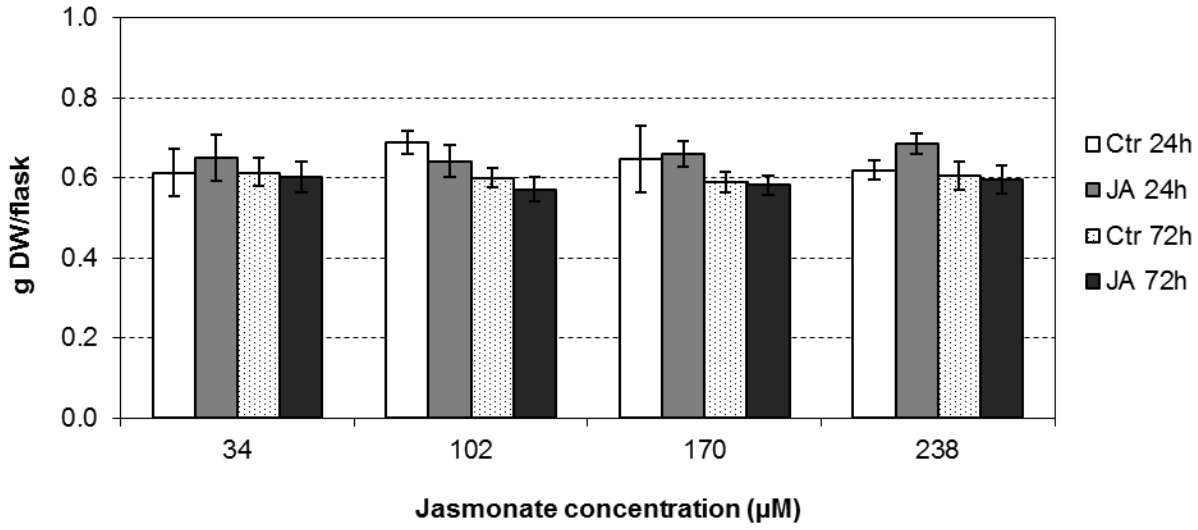

Fig. 4 Cell biomass of *Catharanthus roseus* cell culture (cell-line CRPP) treated with different concentrations of jasmonic acid. Error bars represent standard deviations obtained from three replicates.

statistically significant different to the control ($P < 0.05$). Tabersonine level had highest increment at 24 hours after elicitation of 102 μM (3.6-fold) and 170 μM (3.8-fold) JA. Catharanthine levels had increased about twofold at 24 hours and 72 hours after 102 μM JA elicitation ($P < 0.05$). These results indicate that the flux of metabolites to TIA is clearly enhanced by JA elicitation. Several studies have shown that exogenous application of jasmonates enhances TIA production (Rijhwani and Shanks 1998; El-Sayed and Verpoorte 2002, 2004; Lee-Parsons et al. 2004; Lee-Parsons and Royce 2006; Vázquez-Flota et al. 2009) and the induction is associated with the elevated expression of a set of genes involved in TIA biosynthesis such as geraniol synthase (Simkin et al. 2013), geraniol 10-hydroxylase or also known as geraniol 8-oxidase (Collu et al. 2001), anthranilate synthase, D-1-deoxyxylulose 5-phosphate synthase, tryptophan decarboxylase, strictosidine synthase, and strictosidine β-D-glucosidase (Memelink et al. 2001). Our present study also shows that the TIA levels did not significantly increase in a JA concentration dependent way ($P < 0.05$), and that the differences in responses between biological replicates can be quite high. The central TIA intermediate strictosidine is by far the most abundant compound of the TIA pathway. Serpentine is present at about 20 times lower level, whereas tabersonine and catharanthine levels were two orders of magnitude lower. Small changes in strictosidine levels may thus cause larger effects on these minor alkaloids. But in fact the changes observed may in relative terms be similar to those observed for strictosidine, in terms of absolute amounts they represent a very low percentage of the precursor strictosidine.

Combination treatment of geraniol feeding and JA elicitation

The combination treatment of geraniol feeding and JA elicitation could affect both precursor availability and TIA formation. In a previous study using the Leiden *C. roseus* A11 cell line, Lee-Parsons and Royce (2006) reported that feeding geraniol (0.5 mM) doubled the ajmalicine level if compared to the non-fed cells. However when feeding geraniol (0.1 – 1.0 mM) a day after elicitation, the ajmalicine production was not significantly affected compared to jasmonate elicited cells. As gene activation and transcription to enzymes occurs within minutes to a few hours upon jasmonate elicitation (Menke et al. 1999, van der Fits and Memelink 2001), the addition of the precursor may result in no significant changes in product accumulation when applied a day after elicitation. Therefore, in the present study we tested the reverse approach by adding jasmonic acid a day (day 5) after geraniol feeding (day 4).

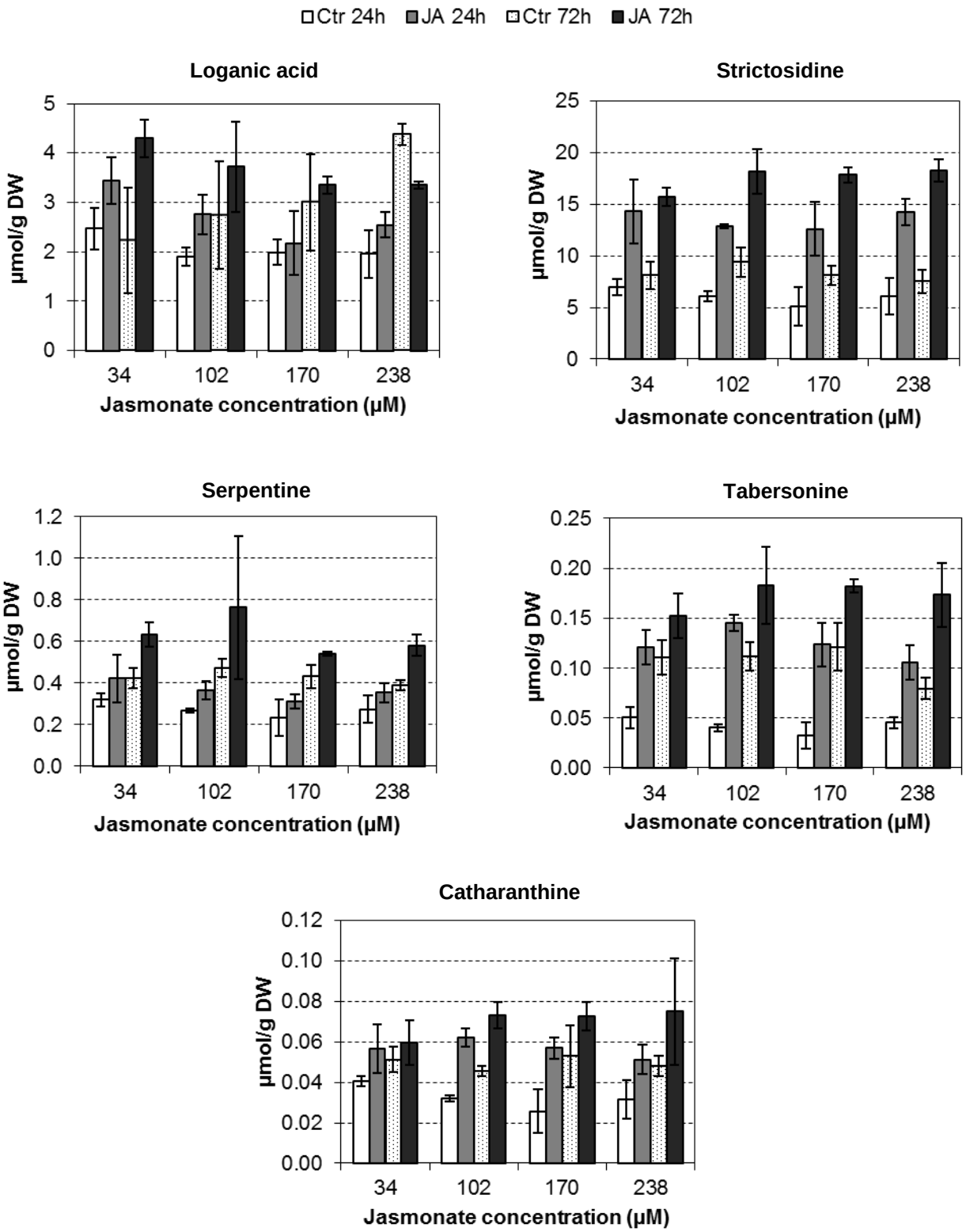

Fig. 5 Accumulation of some compounds in *Catharanthus roseus* cell culture (cell-line CRPP) treated with different concentrations of jasmonic acid. Error bars represent standard deviations obtained from three replicates.

Based on the earlier experiments, the combination treatment of 1.62 mM geraniol and 102 μM JA concentrations was chosen because that geraniol concentration resulted in high accumulation of loganic acid and the new compound, whereas the concentration of 102 μM JA was used because it seems to have a better induction of TIA accumulation.

Figure 6 shows the effect of geraniol and jasmonic acid combination treatments on the accumulation of the *C. roseus* cell (cell-line CRPP) biomass. The biomass accumulation was lower after feeding 1.62 mM geraniol (final concentration). At day 8 after inoculation (96 hours after geraniol feeding), the cell DW was 27% and 20% lower in geraniol feeding and geraniol + JA treatment, respectively as compared to their controls. This result shows that the additional JA elicitation does not further reduce growth of the cell cultures beyond the effect of the fed geraniol, as discussed in the previous paragraph. Despite the reduced biomass accumulation, the appearance of the geraniol-fed cell cultures with or without added JA seemed comparable to the control cultures, as mentioned before for the geraniol treatment. Analysis of geraniol in the *C. roseus* culture medium showed that it was no longer present in the liquid medium after 24 hours (**Supplement 1**), while also no geraniol was detected in the cell cultures at any of the time-point. The rapid disappearance of geraniol from the liquid medium of cell cultures in 24 hours is also found by Carriere et al. (1989), who indicated that the disappearance of geraniol is mainly due to metabolization into other products rather than being lost by evaporation. The combination treatment confirms the capacity of the CRPP cells to take up and metabolize a high concentration of geraniol without strong adverse effects on biomass accumulation, and this makes it a useful model cell line to study the geraniol and iridoid pathways, including the effects of signaling molecules like JA.

Figure 7 shows the effects of geraniol and the combination treatment with jasmonic acid on loganic acid and TIA at different time-points. Loganic acid was clearly increased (8- to 10-fold compared to the control) at 24 hours (day 5) after geraniol feeding ($P < 0.05$). At day 6, loganic acid remained at high level in the geraniol-fed cells, while it decreased to control levels in the geraniol-fed cells treated with jasmonic acid at 24 hours after elicitation. Strictosidine production was not affected in geraniol feeding, but upon subsequent JA elicitation, strictosidine was increased at 24 hours after elicitation compared to the solvent controls and reached the highest level (30.6 μmol/g DW) after 72 hours (day 8). Though a precise mass balance cannot be made, the decrease in loganic acid is followed by an increase of strictosidine in geraniol + JA treatment. Interestingly, the increasing level in geraniol + JA treatment was 68% higher ($P < 0.05$) than after the single JA elicitation treatment (18.2

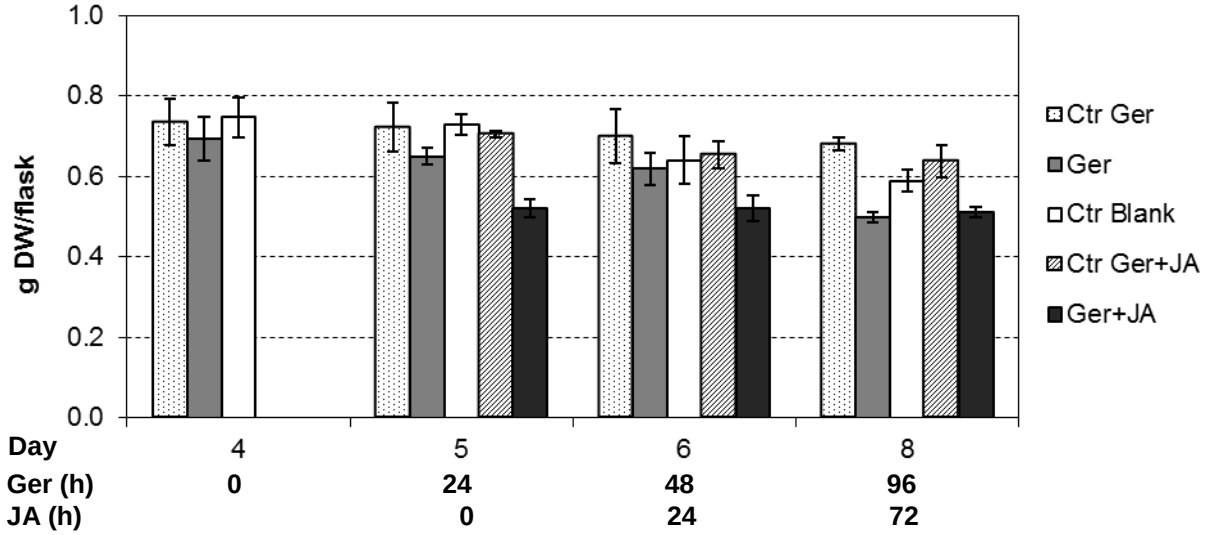

Fig. 6 Cell biomass of *Catharanthus roseus* cell culture (cell-line CRPP) treated with final concentration of 1.62 mM geraniol (Ger) at day 4 and in combination with 102 µM jasmonic acid (JA) added at day 5. Error bars represent standard deviations obtained from three replicates.

µmol/g DW) at the same concentration (102 µM) after 72 hours (see **Fig. 5**). This result shows that a combination of geraniol + JA elicitation specifically increases the flux towards strictosidine. It should be noted that even though strictosidine level did not increase in the only geraniol feeding treatment, the loganic acid accumulation was decreased to the level of the control at day 8 (after 96 hours), suggesting catabolism of loganic acid to other products.

Serpentine levels were not affected significantly after feeding geraniol but they were increased 72 hours after geraniol-fed cells were elicited with jasmonic acid ($P < 0.05$). The serpentine level (0.6 µmol/g DW) at day 8 (72 hours after elicitation) was comparable to the level of serpentine in the single jasmonic acid treatment (see **Fig. 5**).

In the initial study using different concentrations of geraniol, feeding at 1.30 mM and 1.62 mM final concentrations significantly reduced the level of catharanthine and tabersonine ($P < 0.05$). In this study, neither of these compounds was detected in the cells at 96 hours (day 8) after geraniol feeding. Furthermore, subsequent JA elicitation of the geraniol-fed cells did not result in detectable tabersonine and catharanthine accumulation, even though only JA elicitation resulted in higher levels of both compounds (**Fig. 5**). In a previous study, Morgan and Shanks (2000) found an increase of tabersonine levels after feeding geraniol to the *C. roseus* hairy roots cultures. However, the results may not be comparable because Morgan and Shanks (2000) used a much lower concentration of geraniol (0.05 – 0.10 mM) and the root

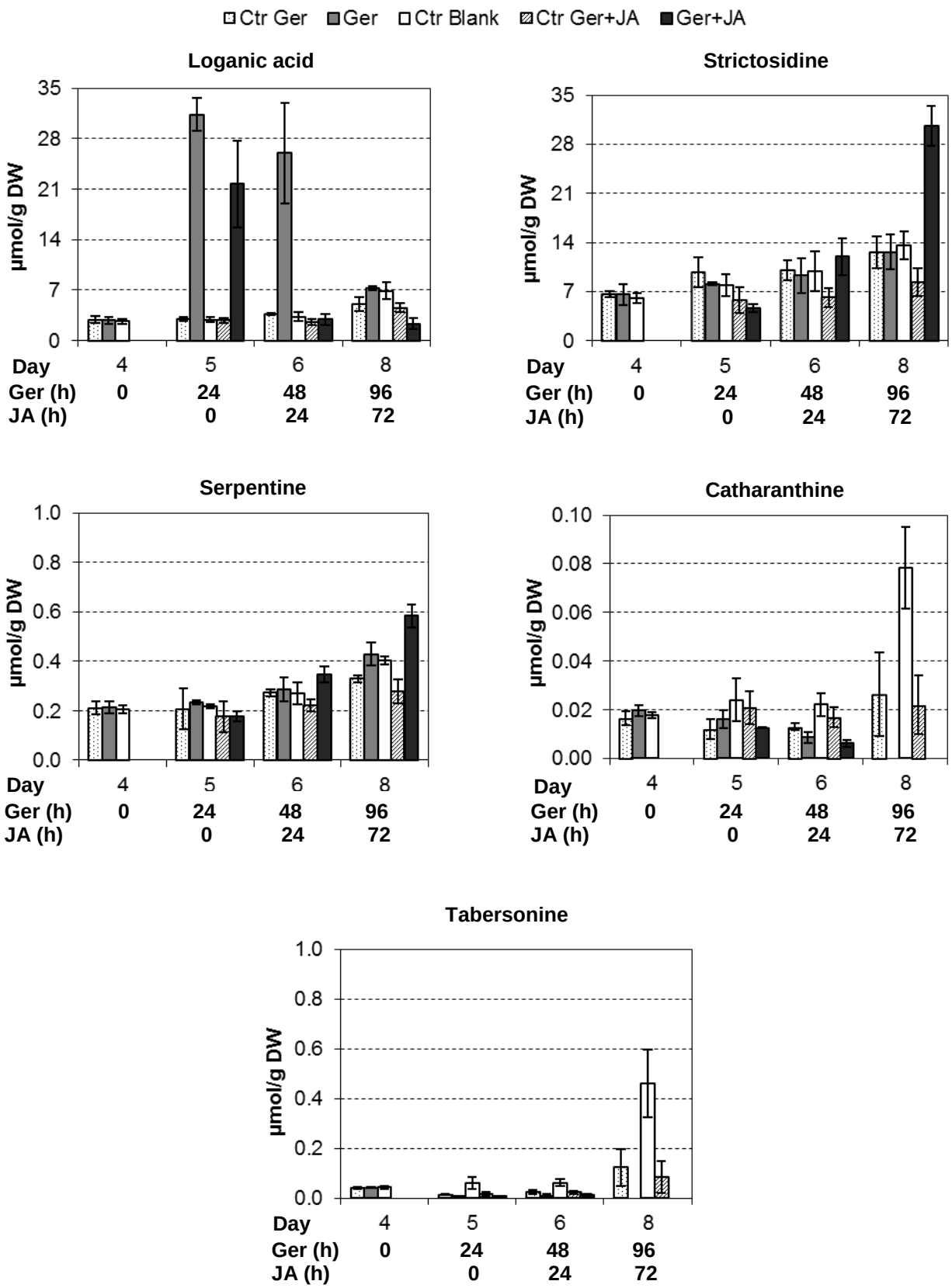

Fig. 7 Accumulation of some compounds in *Catharanthus roseus* cell culture (cell-line CRPP) treated with final concentration of 1.62 mM geraniol (Ger) at day 4 and in combination with 102 µM jasmonic acid (JA) added at day 5. Error bars represent standard deviations obtained from three replicates.

cultures may have responded differently in their uptake and metabolism than our cell suspension cultures. It may be interesting to test the combination of JA elicitation and lower concentrations of geraniol feeding on the TIA and precursors productions in our CRPP cell-line, in which continuous or repetitive feeding could be considered to deliver equal amounts of carbon units over a longer feeding-period as alternative to the high single spiking approach currently employed.

Apart from a negative influence on tabersonine and catharanthine accumulation by high levels of geraniol, it should be noted that tabersonine and catharanthine levels in the control solvent samples (50% v/v ethanol for geraniol feeding, 50% + 20% v/v ethanol for geraniol + JA treatment) were also lower compared to the control blank samples. This indicates that there is probably an additional effect of ethanol to catharanthine and tabersonine production.

As the changes in loganic acid levels after geraniol feeding was not compensated by an increase of known iridoid intermediates (i.e. loganin and secologanin), strictosidine and down-stream TIA, it was interesting to look for new compounds that appeared upon geraniol feeding. Indeed, there is the large unknown peak detected in the HPLC chromatogram of the geraniol-fed samples at the retention time of secologanin, but it had a slightly different UV absorption spectrum if compared to secologanin (see **Fig. 3**). In this case, another HPLC-DAD method was employed to further analyze the broad peak, which resulted in an improved separation and detection of several new peaks subsequently labeled as A, B, C, D and E (**Fig. 8**). **Figure 9** shows the peaks had different UV absorption spectra if compared to loganic acid, secologanin, and strictosidine. The transient levels of those peaks after feeding geraniol and in combination with JA elicitation are shown in **Fig. 10**. Upon geraniol feeding, peaks A and B increased over time followed by a decrease of loganic acid. However, peaks C and D levels are quite stable over time. The level of peak A and B at JA elicitation was about 2 times lower and higher, respectively, than that of the only geraniol feeding. At 24 hours after JA elicitation, peak A was again about similar to only geraniol feeding, whereas the level of peak B was about double than that of the geraniol feeding but did not increase any further in the next period up to 96 hours. Loganic acid level was remarkably decreased at 24 hours after elicitation and remained at the same level at 96 hours, but this level was about 3 times lower than that of the only geraniol feeding. Peak C was about doubled at JA elicitation compared to the only geraniol feeding, the level maintained at 24 hours but subsequently reduced about half at 72 hours (day 8) compared to the initial level. Peak D was about the same level after

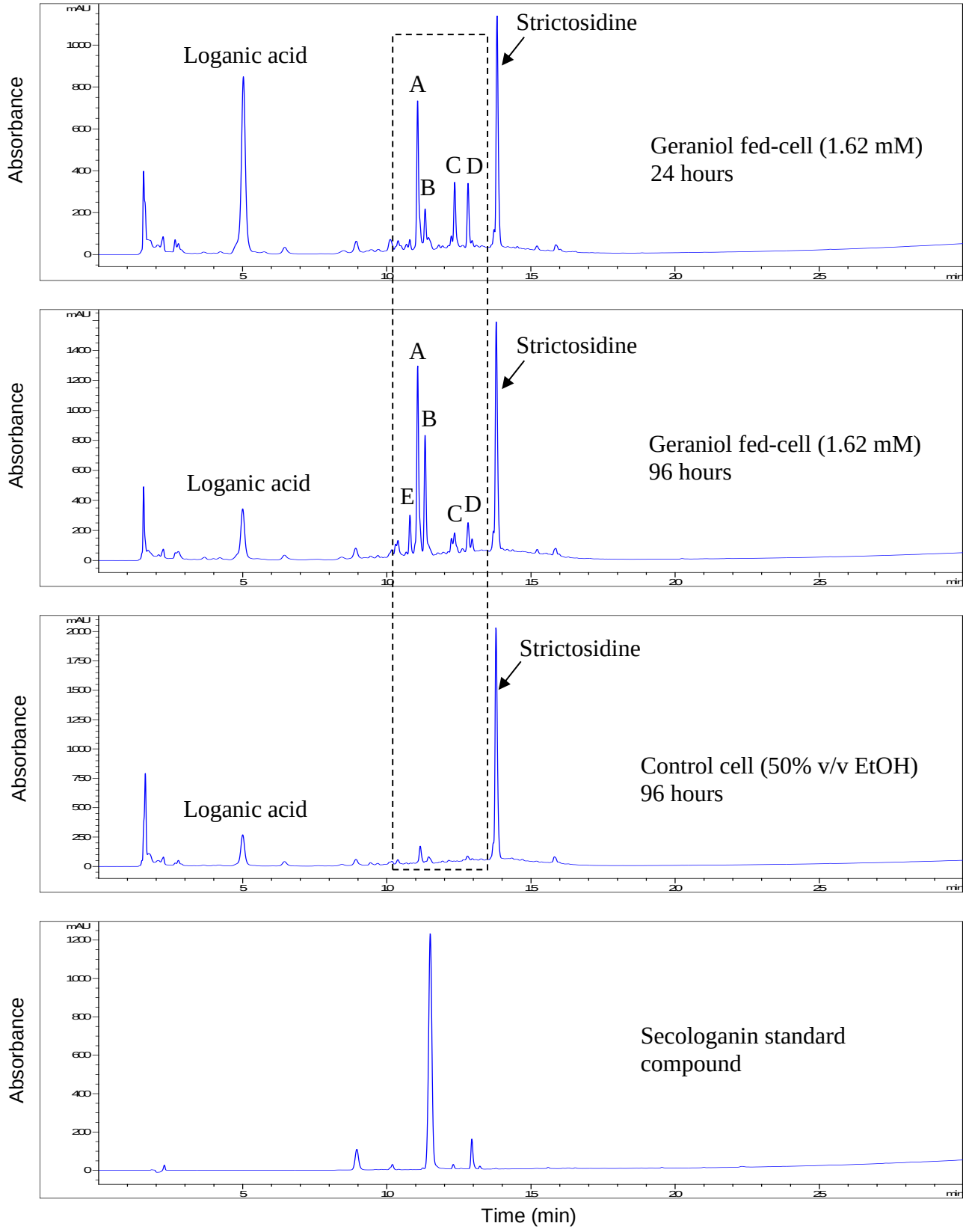

Fig. 8 Chromatograms of HPLC-Diode Array Detector (DAD) of *Catharanthus roseus* cell culture (cell-line CRPP) at 24 and 96 hours after geraniol feeding (1.62 mM), control cell after 96 hours, and standard compound of secologanin. The new peaks labelled as A, B, C, D, and E is detected in the geraniol-fed cells.

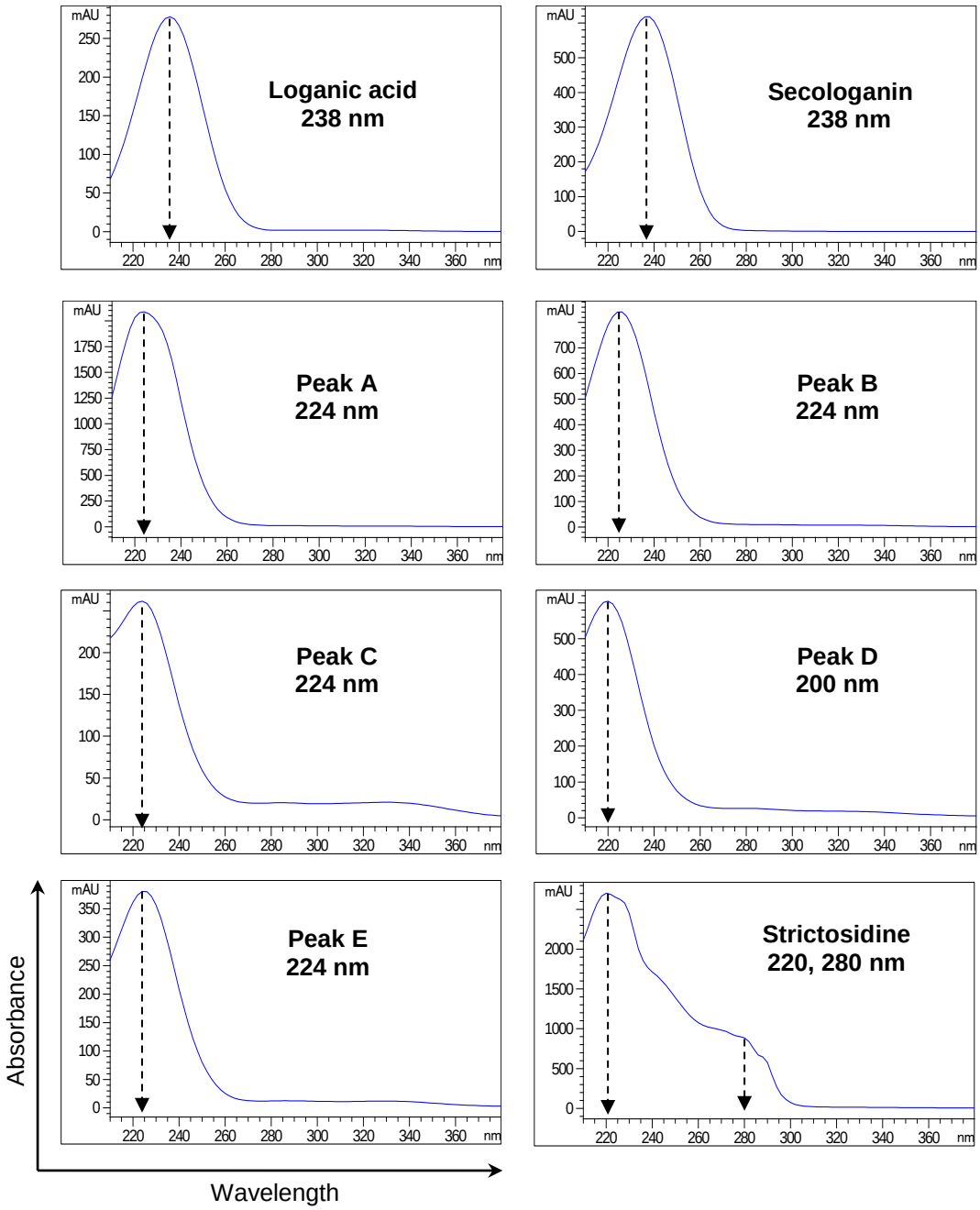

Fig. 9 UV absorption spectra of standard compounds loganic acid, secologanin, strictosidine, and the new peaks (labelled as A, B, C, D, and E) detected geraniol-fed (1.62 mM) *Catharanthus roseus* cell culture (cell-line CRPP). X-axis is wavelength (nm) and Y-axis is absorbance (mAU).

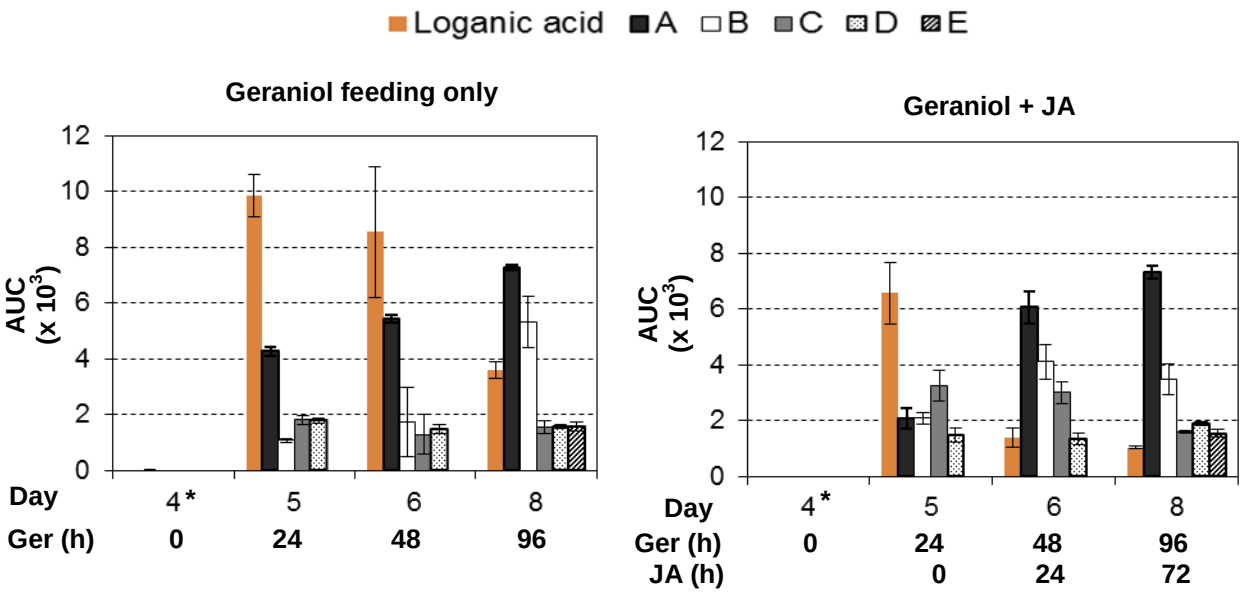

Fig. 10 Relative levels of peak A, B, C, D, E, and loganic acid in *Catharanthus roseus* cell cultures (cell-line CRPP) fed with 1.62 mM geraniol (Ger) at day 4. Geraniol + JA is combination of Ger feeding (1.62 mM) at day 4 and jasmonic acid (JA) elicitation (102 µM) at day 5. Error bars represent standard deviations obtained from three replicates. AUC is area under the curve value determined by HPLC-Diode Array Detector (DAD). *Samples at day 4 are not analyzed.

elicitation, while peak E was only detected at 96 hours after geraniol feeding and it was not affected by JA treatment. The structures of these compounds remain to be determined using further spectroscopic analyses.

The samples of geraniol feeding and the control were analyzed using NMR spectroscopy. Several proton signals appeared in the spectra of the extracts of the treated cells which are not present in the control (**Fig. 11).** Based on the ^{1}H- and 2D-NMR spectra (**Supplement 2 – 6**), the compounds might be analogues of geraniol due to the characteristic signals at δ 6.98 (*t*, *J* = 7.6 Hz), δ 1.87 (*s*), and δ 2.28 (*m*) which have correlation to carboxyl groups at C-8 (HMBC: δ 170). In addition, the doublets at δ 5.56 – δ 5.57 (*J* = 8.0 Hz) and multiplets at δ 3.48 – δ 3.51 indicates the signals of glycosides. However, the complete structure of the compounds are not yet clear and cannot be deduced from the spectrum because of the complexity of the mixture. Further work should be done to isolate the compounds by preparative HPLC. After recovery of the pure compounds, structure elucidation by NMR- and MS-spectroscopy should lead to the complete structures.

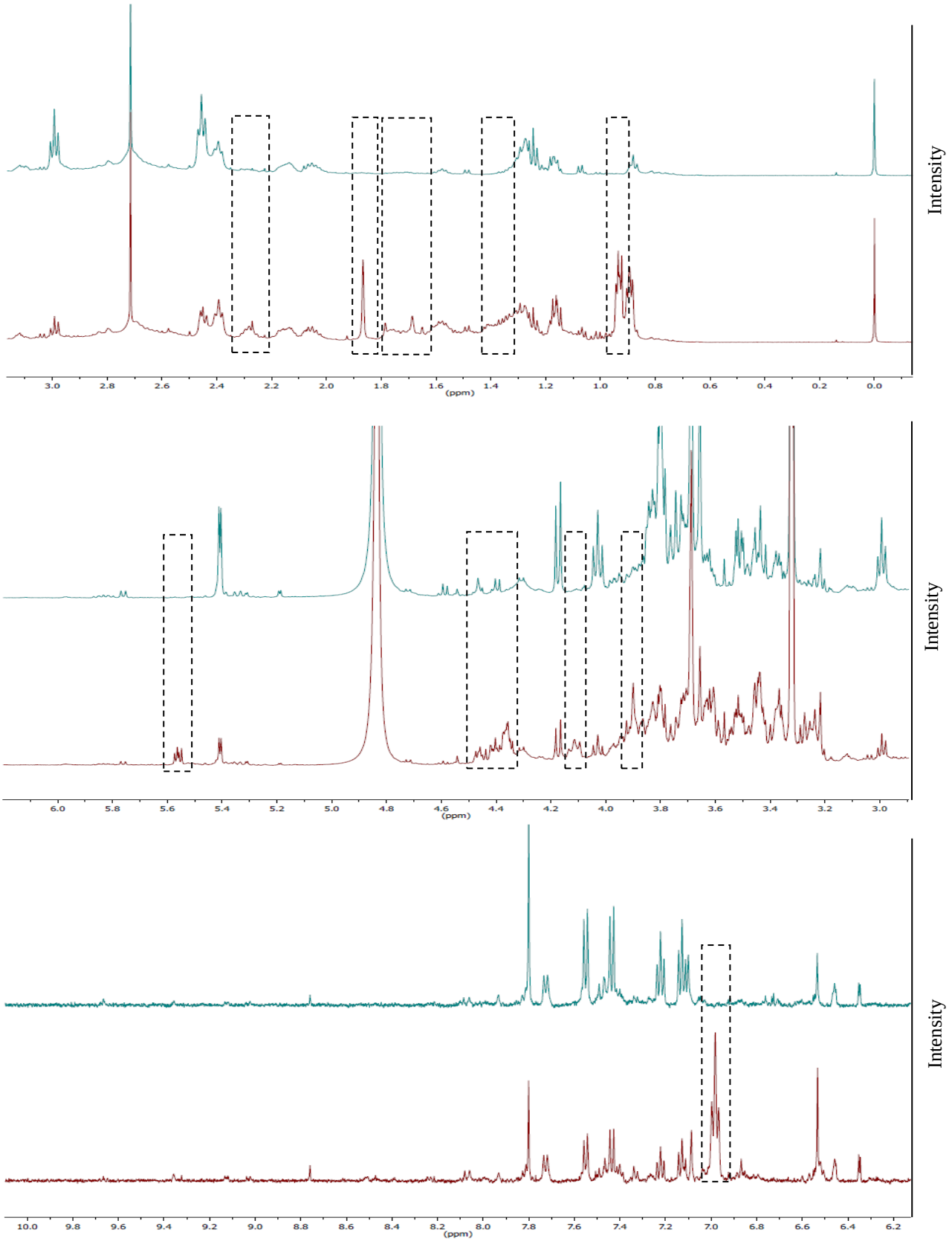

Fig. 11 ^{1}H-NMR spectra of geraniol-fed (brown) and control (blue) cell suspension cultures of *Catharanthus roseus*. Several new ^{1}H signals present in the geraniol-fed cell (brown).

Conclusion

The *C. roseus* CRPP cell line can sustain a high concentration of geraniol without strong adverse effects on biomass accumulation, thus it is a useful system to study the geraniol and iridoid pathway, and the effects of plant signaling molecules such as JA. Feeding the final concentration of 1.62 mM geraniol resulted in high accumulation of loganic acid and some geraniol analogues, but might repress TIA production. Jasmonic acid elicitation increases all TIA compounds detected in the cell suspension culture. A combination of geraniol feeding with subsequent JA elicitation increased the strictosidine level, but not the down-stream TIA if compared to the only elicitation treatment. Further studies on the flow of carbon in different metabolic pathways need to be established as in the current study the measured values only minus catabolism. Moreover, different ranges of geraniol concentrations need to be explored for further optimization of TIA production. In addition, the optimal timing of feeding and elicitation must be established in follow-up studies.

Acknowledgements

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References

Carriere F, Gil G, Tapie P, Chagvardieff P (1989) Biotransformation of geraniol by photoautotrophic, photomixotrophic and heterotrophic plant cell suspensions. Phytochemistry 28: 1087–1090

Contin A, van der Heijden R, Lefeber AWM, Verpoorte R (1998) The iridoid glucoside secologanin is derived from the novel triose phosphate/pyruvate pathway in a *Catharanthus roseus* cell culture. FEBS Lett 434: 413–416

El-Sayed MA, Verpoorte R (2002) Effect of phytohormones on growth and alkaloid accumulation by a *Catharanthus roseus* cell suspension culture fed with the alkaloid precursors tryptamine and loganin. Plant Cell Tiss Organ Cult 68: 265– 270

El-Sayed MA, Verpoorte R (2004) Growth, metabolic profiling and enzymes activities of *Catharanthus roseus* seedlings treated with plant growth regulators. Plant Growth Reg 4: 53–58

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:155–158

Krueger RJ, Carew DP (1978) *Catharanthus roseus* tissue culture: the effects of precursors on growth and alkaloid production. J Nat Prod 41: 327–331

Lee-Parsons CWT, Ertürk S, Tengtrakool J (2004) Enhancement of ajmalicine production in *Catharanthus roseus* cell cultures with methyl jasmonate is dependent on timing and dosage of elicitation. Biotechnol Lett 0: 1595–1599

Lee-Parsons CWT, Royce AJ (2006) Precursor limitations in methyl jasmonate-induced *Catharanthus roseus* cell cultures. Plant Cell Rep 25: 607–612

Memelink J, Kijne JW, van der Heijden R, Verpoorte R (2001) Genetic modification of plant secondary metabolite pathways using transcriptional regulators. Adv Biochem Eng Biotechnol 72: 103–125

Menke FL, Champion A, Kijne JW, Memelink J (1999) A novel jasmonate- and elicitor-responsive element in the periwinkle secondary metabolite biosynthetic gene Str interacts with a jasmonate- and elicitor-inducible AP2-domain transcription factor, ORCA2. EMBO J. 18: 4455–4463

Moreno PRH, van der Heijden R, Verpoorte R (1993) Effect of terpenoid precursor feeding and elicitation on formation of indole alkaloids in cell suspension cultures of *Catharanthus roseus*. Plant Cell Rep 12: 702–705

Morgan JA, Shanks JV (2000) Determination of metabolic rate-limitations by precursor feeding in *Catharanthus roseus* hairy root cultures. J Biotechnol 79:137–145

Rijhwani SK, Shanks JV (1998) Effects of elicitor dosage and exposure time on biosynthesis of indole alkaloids by *Catharanthus roseus* hairy root cultures. Biotechnol Prog 14: 442–449

Saiman MZ, Mustafa NR, Pomahocova B, Verberne M, Verpoorte R, Choi YH, Schulte AE (2014) Analysis of metabolites in the terpenoid pathway of *Catharanthus roseus* cell suspensions. Plant Cell Tiss Organ Cult 117: 225–239

Tikhomiroff C, Jolicoeur M (2002) Screening of *Catharanthus roseus* secondary metabolites by high-performance liquid chromatography. J Chromatogr A 955: 87–93

Whitmer S, Canel C, Hallard D, Gonçalves C, Verpoorte R (1998) Influence of precursor availability on alkaloid accumulation by transgenic cell line of *Catharanthus roseus*. Plant Physiol 116: 853–857

Whitmer S, van der Heijden R, Verpoorte R (2002) Effect of precursor feeding on alkaloid accumulation by a strictosidine synthase over-expressing transgenic cell line S1 of *Catharanthus roseus*. Plant Cell Tiss Organ Cult 69: 85–93

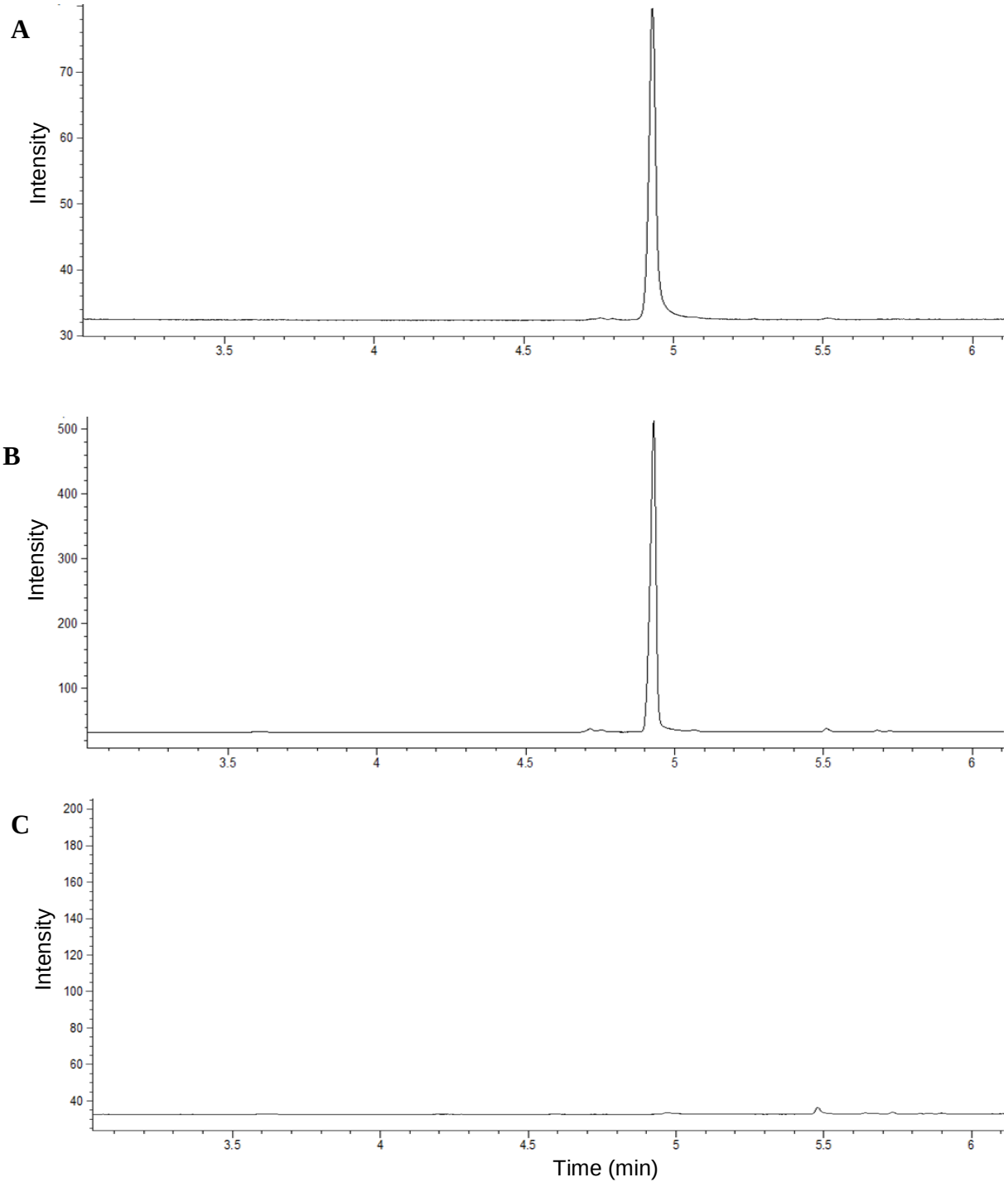
van der Fits, Memelink J (2001) The jasmonate-inducible AP2/ERF-domain transcription factor ORCA3 activates gene expression via interaction with a jasmonate-responsive promoter element. Plant J 25: 43–53

van der Heijden R, Jacobs DI, Snoeijer W, Hallard D, Verpoorte R (2004) The *Catharanthus* alkaloids: pharmacognosy and biotechnology. Curr Med Chem 11: 607– 628

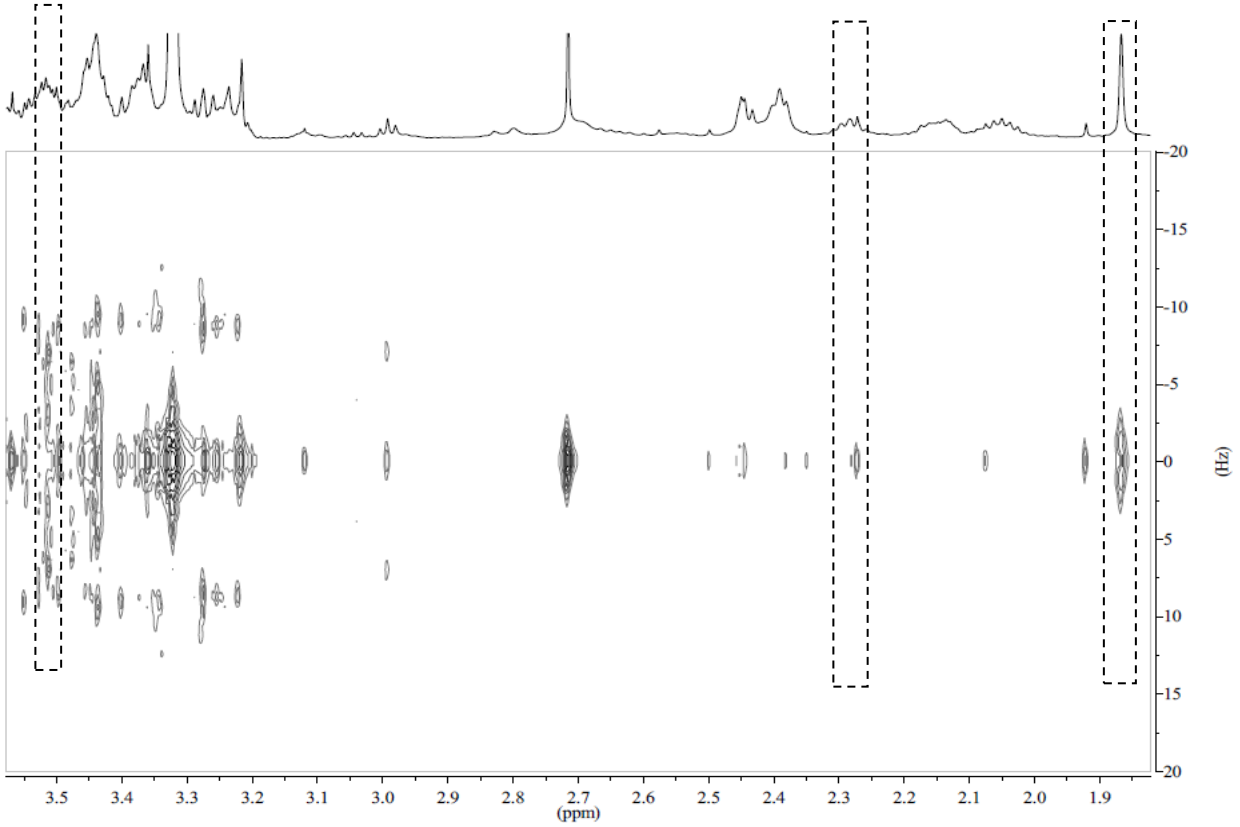
Vázquez-Flota F, Hernández-Domínguez E, Miranda-Ham MdL, Monforte-González M (2009) A differential response to chemical elicitors in *Catharanthus roseus* in vitro cultures. Biotechnol Lett 31: 591–595

Zhao J, Hu Q, Guo YQ, Zhu WH (2001) Effects of stress factors, bioregulators, and synthetic precursors on indole alkaloid production in compact callus clusters cultures of *Catharanthus roseus*. Appl Microbiol Biotechnol 55: 693–698

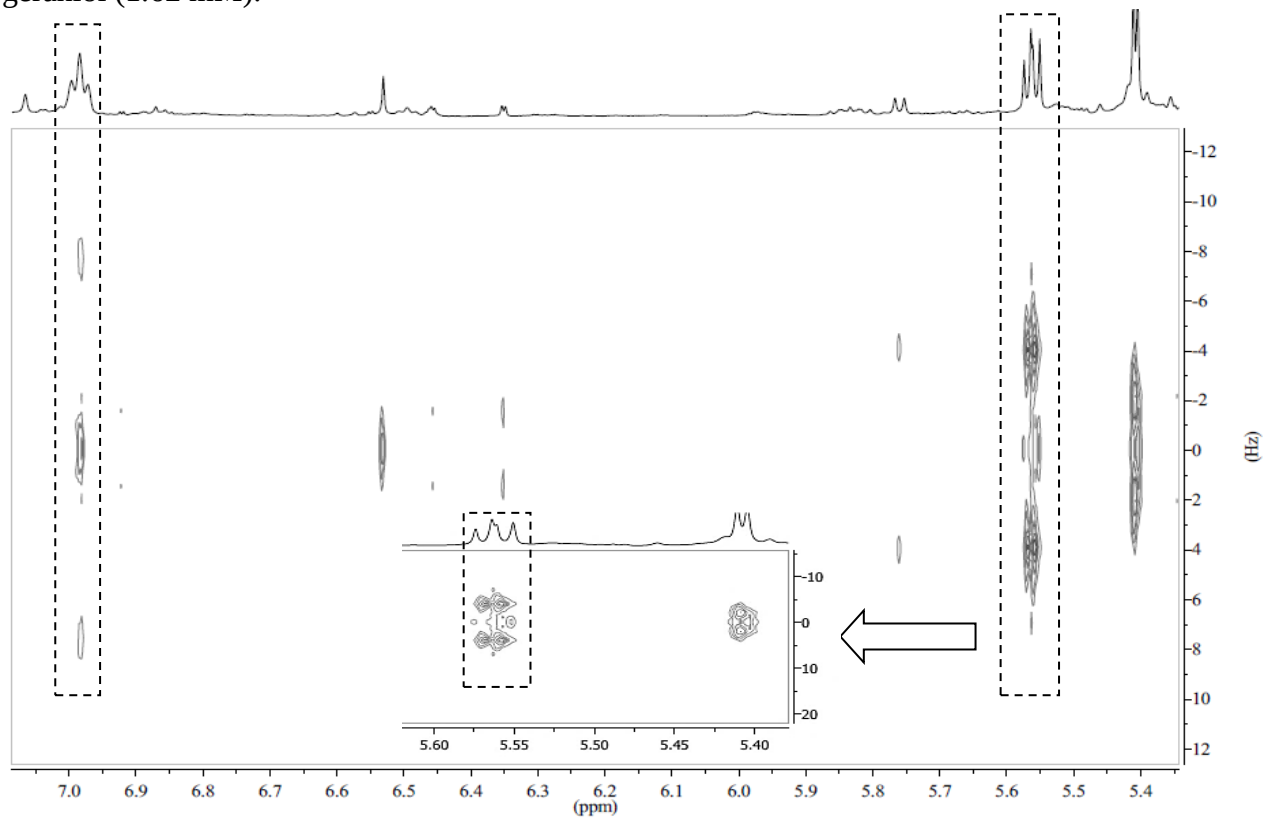
Supplement 1 Chromatograms of GC-Flame Ionization Detector (FID) of geraniol standard compound (A), and liquid medium of *Catharanthus roseus* cell suspensions at 0 hour (B) and 24 hours (C) after geraniol feeding (1.62 mM), respectively.



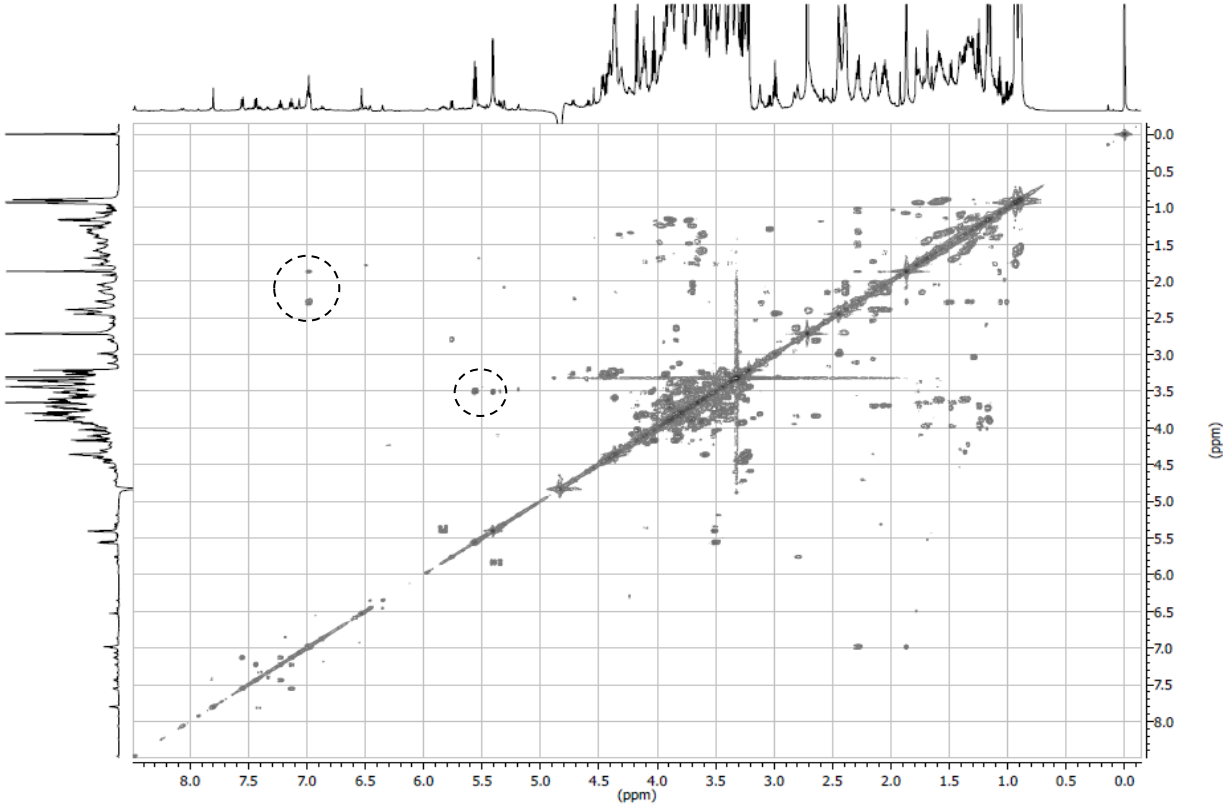
Supplement 2 *J*-resolved ^{1}H-NMR (δ 1.85 – δ 3.55) of *Catharanthus roseus* cells fed with geraniol (1.62 mM)



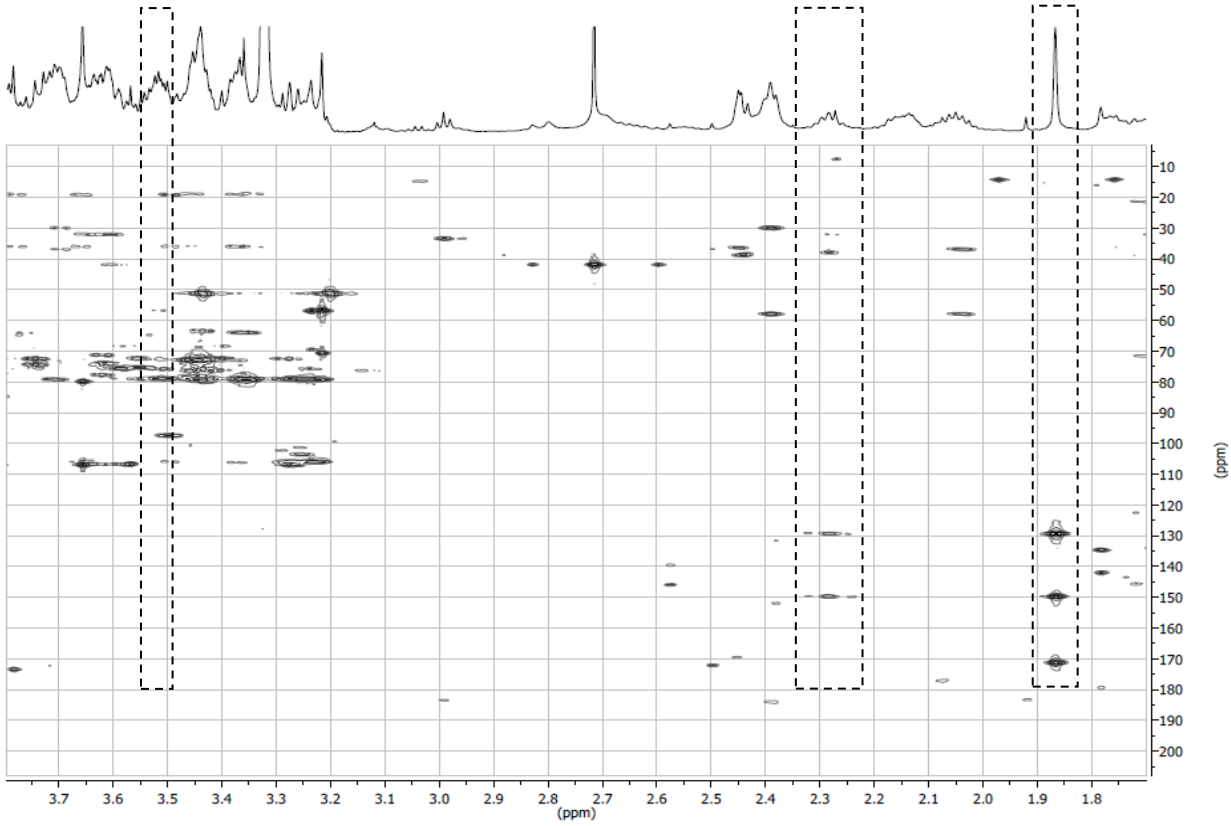
Supplement 3 *J*-resolved ^{1}H-NMR (δ 5.35 – δ 7.05) of *Catharanthus roseus* cells fed with geraniol (1.62 mM).



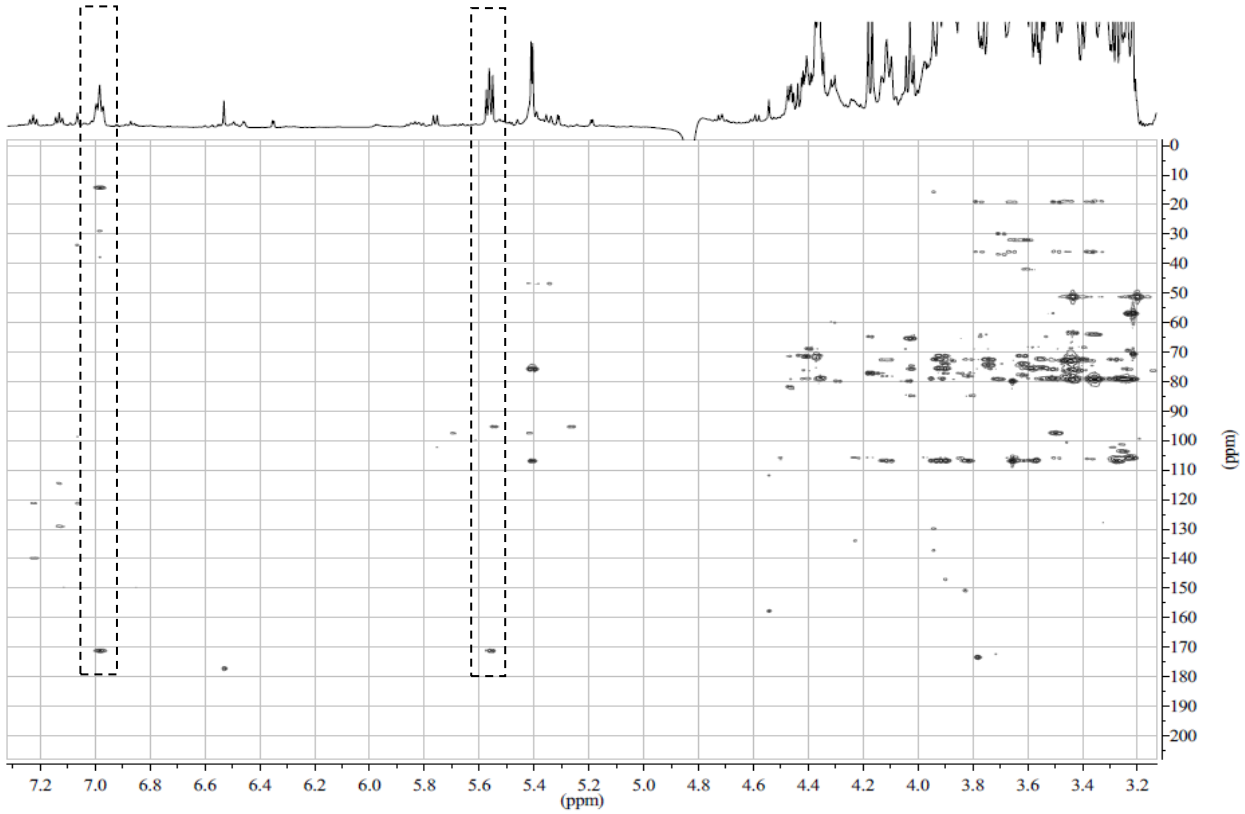
Supplement 4 COSY spectrum (δ 0.00 – δ 8.50) of *Catharanthus roseus* cells fed with geraniol (1.62 mM).



Supplement 5 HMBC spectrum (δ 1.70 – δ 3.80) of *Catharanthus roseus* cells fed with geraniol (1.62 mM).



Supplement 6 HMBC spectrum (δ 3.20 – δ 7.30) of *Catharanthus roseus* cells fed with geraniol (1.62 mM).



Chapter

6

Catharanthus roseus cell suspensions overexpressing geraniol synthase in the plastid and cytosol

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Abstract

Previous studies showed that geraniol, an upstream precursor in the monoterpenoid pathway, could be a limiting factor for terpenoid indole alkaloid (TIA) production in *Catharanthus roseus* cells and hairy root cultures. This shortage in precursor availability could be due to limited expression of the geraniol synthase gene, and consequently a low activity of the encoded enzyme, which is located in the plastid and catalyzes the conversion of geranyl diphosphate to geraniol, or to the limitation of geraniol transport from the plastid to the cytosol. In the present study, *C. roseus* geraniol synthase was overexpressed in the plastid (CrGES) and cytosol (ΔplCrGES) of a non-TIA producing *C. roseus* cell culture via biolistic transformation. Transient expression studies confirmed the localization of the enzyme in the targeted subcellular compartments. Expression of the geraniol synthase was verified by Northern blot and reverse transcription-PCR. Neither TIA nor iridoid precursors were detected by HPLC-DAD in the transformed *C. roseus* cell cultures. Nevertheless, metabolomics analysis by NMR shows that the levels of phenylalanine, tyrosine, valine, and leucine were significantly higher in the CrGES overexpressing lines, while being lower in the ΔplCrGES overexpressing lines, compared to the controls. Isoleucine and tryptophan levels were also higher in the CrGES than the ΔplCrGES overexpressing lines. Apparently, overexpression of CrGES in the plastids and ΔplCrGES in the cytosol caused different effects on several primary metabolites including some derived from the shikimate pathway.

Introduction

Catharanthus roseus (Madagascar periwinkle) is a medicinal plant which produces a class of secondary metabolites called terpenoid indole alkaloid (TIA). More than 130 TIAs have been isolated from *C. roseus* and some of them are important pharmaceuticals such as the antihypertensive drugs ajmalicine and serpentine, and the antineoplastic agents vinblastine and vincristine (van der Heijden et al. 2004). However, most TIAs are produced at low levels in the plant, particularly the valuable chemotherapy drugs vinblastine and vincristine which are present only in trace amounts, resulting in high market price.

Biotechnological approaches using *in vitro* cell and tissue cultures of *C. roseus* have been developed as an alternative source of TIA. However, a high producing cell line has not been obtained despite all efforts in the optimization of growing and production conditions (Moreno et al. 1995). Although the mass cultivation of *C. roseus* cells is feasible in a large-scale bioreactor, the cost of production of alkaloids is too high for commercialization

(Verpoorte et al. 2000). Metabolic engineering by overexpressing the biosynthetic genes of the target pathway or suppressing flux of competing pathways are promising approaches to improve the production of TIA in *C. roseus* cell cultures (Verpoorte et al. 2000; Verpoorte et al. 2002; Zhao and Verpoorte 2007). Single or multiple genes encoding the biosynthesis enzymes of the TIA pathway have successfully been overexpressed (anthranilate synthase: AS, 1-deoxy-D-xylulose synthase: DXS, tryptophan decarboxylase: TDC, strictosidine synthase: STR, geraniol 8-oxidase: G8O (also known as geraniol 10-hydroxylase: G10H), desacetoxyvindoline 4-hydroxylase: DAT, apoplastic peroxidase: CrPrx) in *C. roseus* cells (Canel et al. 1998), hairy roots (Magnota et al. 2007; Peebles et al. 2010; Wang et al. 2010; Jaggi et al. 2011), and plants (Pan et al. 2012). In addition, overexpression of the transcription factors ORCA2 (Liu et al. 2011) and ORCA3 (van der Fits and Memelink 2000; Peebles et al. 2009; Wang et al. 2010; Pan et al. 2012), and an ATP-binding cassette (ABC) transporter (Pomahačová et al. 2009) were studied in *C. roseus,* which resulted in elevated level of some TIAs. However, the precursors from primary metabolism seem to be the limiting factor in increasing production. Channeling of the metabolic flux towards TIA biosynthesis seems thus on important target for metabolic engineering to improve TIA production and to reduce the production costs with plants or plant cell cultures.

Metabolic engineering requires knowledge on the biosynthesis pathway of the products of interest, the subcellular compartmentation of specific steps in the pathway, and the transport of the intermediates between intracellular compartments and between different cell types. The biosynthesis of TIA in *C. roseus* is a complex metabolic pathway requiring precursors from two different biosynthetic routes, i.e. tryptamine from the shikimate/tryptophan pathway and secologanin from the iridoid pathway. Condensation of tryptamine and secologanin produces strictosidine, the central intermediate of TIAs. Among the two precursor pathways, the iridoid pathway is considered a major rate-limiting factor for TIA production in *C. roseus* cell cultures (Whitmer et al. 2002; van der Heijden et al. 2004; Zhao and Verpoorte 2007).

The iridoid precursors of the TIA derive from 8-hydroxygeraniol (also known as 10-hydroxygeraniol) which is formed upon hydroxylation of geraniol generated from geranyl diphosphate (GPP). Geranyl diphosphate is a condensation product of the basic isoprene units, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). In plants, IPP and DMAPP are produced via two different metabolic pathways each leading to a distinct set of terpenoid derivatives. These two pathways closely interact but they are separated on

subcellular level, i.e. the cytosolic/peroxisomal mevalonate pathway (MVA) and the plastidial methyl-erythritol phosphate (MEP) pathway (Rohmer 1999; Sapir-Mir et al. 2008; Simkin et al. 2011; Pulido et al. 2012). The iridoid/terpenoid moiety of TIA derives from GPP produced via the MEP pathway (Contin et al. 1998). This intermediate is primarily produced by the plastidial enzyme geranyl diphosphate synthase (GPPS), but can also be released as an intermediate during the formation of farnesyl diphosphate (FPP) catalyzed by FPP synthase (FPPS), which is localized in the peroxisome/cytosol (Martin et al. 2007; Thabet et al. 2011). Although the GPP production in the mevalonate pathway remains unclear, a few studies indicate that a low GPP pool was available in the cytosol for the synthesis of limonene in transgenic *Nicotiana tabacum* (Wu et al. 2006) and geraniol in transgenic *N. benthamiana* (Dong et al. 2013).

Several key biosynthesis enzymes in the TIA pathway have been characterized and overexpressed in *C. roseus.* Recently, the enzyme that catalyzes the conversion of GPP into geraniol, i.e. geraniol synthase (CrGES) has been isolated and characterized from *C. roseus* (Simkin et al. 2013). This enzyme is of interest since geraniol is considered as the limiting upstream precursor in TIA biosynthesis; feeding geraniol increased tabersonine and ajmalicine production in *C. roseus* hairy roots (Morgan and Shanks 2000) and cell suspension cultures (Lee-Parsons and Royce 2006), respectively. In the present study, we overexpressed CrGES, a plastidial localized-enzyme, in *C. roseus* cell cultures via biolistic transformation to increase the overall availability of geraniol for TIA biosynthesis. To evaluate and overcome a possible limitation in transport of geraniol from the plastid to the cytosol or other limitations in the plastidial MEP pathway, ΔplCrGES was expressed in the cytosol by removing the plastidial leader peptide in CrGES. It is conceivable that through ΔplCrGES expression in the cytosol, the mevalonate pathway can be directly linked to the TIA pathway and thus possibly lead to increased TIA biosynthesis by feeding mevalonic acid. In this study, changes in the levels of TIAs, precursors, and metabolites in the transformed *C. roseus* cells were determined.

Materials and methods

Cell culture

Catharanthus roseus cell suspension cultures (cell-line MP183L) were subcultured weekly by transferring 10 ml culture into 50 ml of Linsmaier and Skoog (LS) medium (Linsmaier and Skoog 1965) containing 30 g/l sucrose, 2 mg/l NAA, and 0.2 mg/l kinetin.

The cultures were grown on a gyratory shaker at 120 rpm at 25 ^{o}C in 16/8 hour light/dark regime (20 µE/m^2/sec) at 70% relative humidity.

Cloning, vector constructions and transformation

A full fragment of *C. roseu*s geraniol synthase (CrGES, Genbank ID: JN882024, Simkin et al. 2013, **Supplement 1**) or a truncated version of CrGES lacking the plastidial leader peptide (ΔplCrGES) were produced by a PCR-based strategy. For transient expression, constitutive, and inducible constructs; the forward primers for CrGES and ΔplCrGES were 5'GTCGACAAAATGGCAGCCACAATTAGTAACC-3' and 5'-GTCGACAAAATG TCTCTGCCTTTGGCAACT-3', respectively. The reverse primer for the transient expression study was 5'-GTCGACAAAACAAGGTGTAAAAAACAAAGC-3'; while for constitutive and inducible constructs, the reverse primer was 5'-TCTAGATTAAAAACAAGGTGTA AAAAACAAAGC-3' (**Supplement 2**). Fragments were amplified by PCR (MyCycler Thermal Cycler, Biorad) with following procedures: 98 °C, 1 min; 35 cycles, 98 °C, 15 sec; 57 °C, 20 sec; 72 °C, 1 min; 72 °C, 5 min. The PCR products were first cloned into a pJET1.2/blunt cloning vector (Thermo Scientific, Pittsburgh, PA, USA) and sequenced for confirmation. Subsequently, the verified CrGES and ΔplCrGES fragments were ligated into 1) pTH2-Δ*EcoRI* plasmid excised with *SalI* (transient expression construct, Niwa 2003); 2) pRT101 plasmid excised with *XhoI/XbaI* (constitutive construct, Töpfer et al. 1987); 3) pER8 plasmid excised with *XhoI/SpeI* (inducible construct, Zuo et al. 2000) (**Fig. 1A – C**).

Plasmids containing CrGES and ΔplCrGES were introduced into *C. roseus* cells via biolistic transformation (van der Fits and Memelink 1997). The control cells were transformed with the corresponding plasmid without insert. For transient expression and subcellular localization studies, transformed cells were placed on solid LS medium and viewed after 24 hours using a Zeiss Observer laser scanning microscope equipped with fluorescence filters. The transformed cells with constitutive or inducible expression constructs were cultured on solid LS medium containing 50 µg/ml hygromycin. The individual putative transgenic calli grown on this selective medium were converted to cell suspensions and subcultured every week by transferring 10 ml of cell suspension into 50 ml LS medium containing 30 g/l sucrose, 2 mg/l NAA, 0.2 mg/l kinetin, and 50 mg/l hygromycin.

Estradiol treatment and cell harvesting

At the fifth day after subculturing, the inducer *β*-estradiol (Sigma) dissolved in DMSO was added at a concentration of 10 µM into 10 ml aliquots of the inducible transgene cell suspensions. Control cells were treated with DMSO at a final concentration of 0.1% (v/v). Estradiol-induced cells and controls were harvested 24 hours after the treatments. Unless mentioned otherwise, cells containing constitutive transgenes did not receive any treatment and the cell cultures were harvested one week after subculturing. Harvested cells were immediately frozen in liquid nitrogen. Aliquots of the samples were stored at -80 ^{o}C for RNA extraction and the remaining biomass was lyophilized for 72 hours prior to metabolite analysis.

RNA extraction, Northern blot, reverse transcriptase-PCR

Frozen cells were ground to a fine powder in liquid nitrogen. Total RNA was extracted with two volumes of hot phenol buffer (1:1 mixture of phenol and 100 mM LiCl, 10 mM Na-EDTA, 1% SDS, 100 mM Tris) and one volume of chloroform. RNA was precipitated overnight with LiCl at a final concentration of 2 M, washed twice with 70% ethanol, and suspended in water.

Northern blot analysis was performed as described by Memelink (1994) with some modifications. Ten µg RNA samples were subjected to electrophoresis on 1.5% agarose/1% formaldehyde gels, and blotted to GeneScreen nylon membranes (Perkin-Elmer Life Science, Boston, MA, USA). Blots were prehybridized for several hours in 1M NaCl, 10% dextran sulfate (sodium salt, Sigma), 1% SDS, and 50 µg/ml denatured salmon sperm DNA at 65 ^{o}C before addition of denaturated ^{32}P-labeled DNA probes. After overnight hybridization, blots were washed twice at 42 ^{o}C for 30 min with 0.1 x SSPE (saline/sodium/phosphate/EDTA) and 0.5% SDS. Finally, the blots were washed briefly with 0.1 x SSPE at room temperature. Blots were exposed to X-ray films (Fuji, Tokyo, Japan).

Reverse transcription was carried out using the Revert Aid^{TM} H Minus First Strand cDNA Synthesis Kit (Fermentas) following to the manufacturer's instruction. The cDNA synthesized from each sample was used as template in PCR. Negative controls were performed by excluding reverse transcriptase enzyme in the reaction.

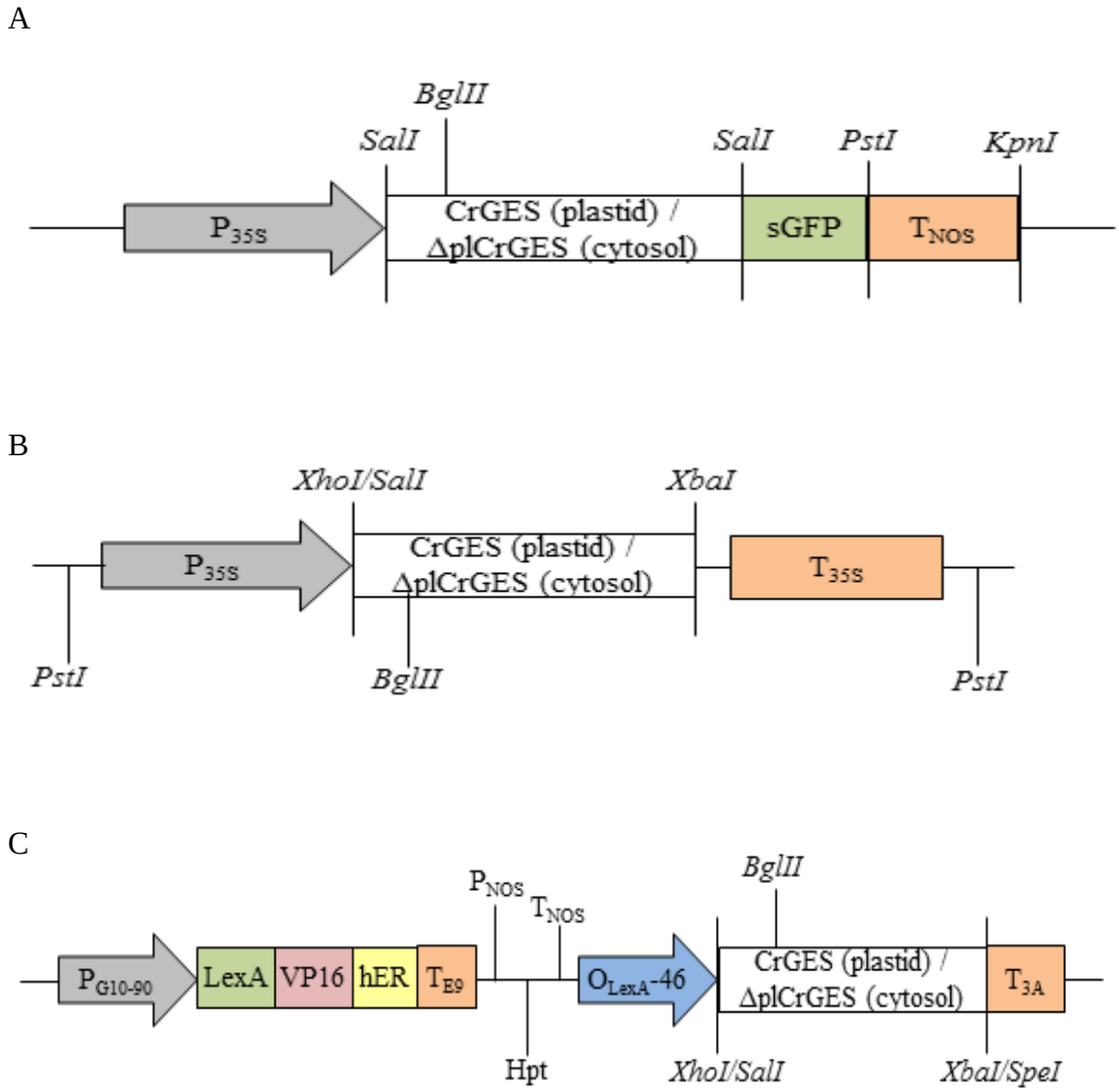

Fig. 1 Schematic representation of the constructs A) pTH2-Δ*EcoRI* (Niwa 2003); B) pRT101 (Töpfer et al. 1987); C) pER8 (Zuo et al. 2000) vectors containing either the full-length fragment of *Catharanthus roseus* geraniol synthase (CrGES) or the truncated CrGES without plastidial leader peptide (ΔplCrGES) that are targeting the enzyme to the plastid or the cytosol, respectively. P_{35S}: cauliflower mosaic virus 35S promoter, sGFP: synthetic green fluorescence protein (S65T), T_{NOS}: nopaline synthase terminator, T_{35S}: cauliflower mosaic virus 35S terminator, $P_{G10\text{-}90}$: a synthetic promoter controlling XVE (Ishige et al. 1999), LexA *E. coli* repressor DNA binding domain, VP16: herpes simplex viral protein 16, hER: hormone-binding domain of human estrogen receptor, T_{E9}: pea rbcS E9 terminator, P_{NOS}: nopaline synthase promoter, Hpt: hygromycin phosphotranferase II coding sequence, O_{LexA}: operator region of LexA promoter, -46: TATA box of 35S promoter, T_{3A}: pea rbcS 3A terminator.

Jasmonic acid elicitation

Jasmonic acid (Sigma) at a final concentration of 100 µM was fed to the cell suspensions containing the constitutive transgenes at the fifth day after subculturing. Subsequently, the treated cultures were harvested at 24, 48, and 72 hours after elicitation. Samples were freeze-dried for 72 hours and stored for TIA analysis.

Analysis of terpenoid indole alkaloids

Freeze-dried cells (100 mg) were extracted with 5 ml methanol, vortexed, sonicated for 20 min, and centrifuged for 30 min (3,500 rpm). The dried supernatant was suspended in 1 ml phosphoric acid (1 M). Samples were subjected for terpenoid indole alkaloid and precursor analysis using high performance liquid chromatography-diode array detector (HPLC-DAD) (Agilent Techologies Inc., Santa Clara, CA, USA) as described by Saiman et al. (2014).

Geraniol analysis

Freeze-dried cells (100 mg) were extracted with 5 ml dichloromethane, vortexed, ultrasonicated 10 min, and centrifuged for 10 min (3,000 rpm, 4 ^{o}C). The eluent was concentrated under a flow of nitrogen and 1 µl of concentrated extract was injected into gas chromatography-mass spectroscopy (GS-MS) (Agilent Technologies Inc.) equipped with a DB-5 capillary column (30 m x 0.25 mm i.d., film thickness of 0.25 um) (J&W Scientific Inc., Folsom, CA, USA). The initial oven temperature was 45 ^{o}C for 1 min, and was increased to 300 ^{o}C at a rate of 10 ^{o}C/min and held for 5 min at 300 ^{o}C. Geraniol standard compound (Sigma) was used for identification.

NMR and multivariate data analysis

Freeze-dried cells (25 mg) were extracted with 1:1 CD_3OD:KH_2PO_4 buffer in D_2O (pH 6.0, containing 0.01% trimethylsilyl propanoic acid [TMSP] as internal standard). The mixture was vortexed for 10 sec, sonicated for 10 min, and centrifuged for 15 min (14,000 rpm). Samples were analyzed using 500 MHz NMR (Bruker, Karlsruhe, Germany). NMR spectra were manually phased, baseline corrected, and calibrated to TMSP at 0.0 ppm using XWIN NMR version 3.5 (Bruker). AMIX software (Bruker) was used for bucketing (width δ 0.04) and data reduction of the ^{1}H-NMR spectra (δ 0.40 – 10.00) using total intensity scaling. Multivariate data analysis was performed with the SIMCA-P+ software version 12.0

(Umetrics, Umeå, Sweden). Analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) was performed on IBM SPSS Statistics 20 (SPSS Inc., Chicago, IL, USA) to determine statistical differences ($P < 0.05$) between means of groups.

Results and discussion

Cloning, vector constructs, and transformation

The geraniol synthase gene of *C. roseu*s (CrGES) was recently characterized by Simkin et al. (2013). It has a 1770 bp DNA sequence and encodes a protein of 589 amino acids in length. CrGES is localized in plastids as suggested by the leader peptide encoded in its cDNA (Simkin et al. 2013). To overexpress CrGES in the plastid and in the cytosol, a full-length CrGES fragment and a truncated version of CrGES lacking the first 156 coding nucleotides (ΔplCrGES) were constructed. Primers were designed for three different vectors employed for different purposes, i.e. pTH2-Δ*EcoRI* for transient expression and subcellular localization studies, pRT101 for constitutive expression, and pER8 for estradiol-inducible transgene expression. The pTH2-Δ*EcoRI* plasmid used in this study is a derivative of pTH2 by adding the "EKE" linker in the *EcoRI* site resulting in loss of the *EcoRI* site and introduction of a *KpnI* site. The pTH2 plasmid has been widely used to study subcellular localization because it encodes the synthetic green fluorescent protein (sGFP: S65T, Niwa 2003). To insert CrGES or ΔplCrGES fragments between the CaMV 35S promoter and sGFP in the pTH2-Δ*EcoRI*, a *SalI* site was introduced at the 5' and 3' of the inserts. For constitutive and inducible expression constructs, CrGES and ΔplCrGES fragments were amplified with a *SalI* site at the 5' and *XbaI* site at the 3', which is compatible with *XhoI/XbaI* and *XhoI/SpeI* sites in the pRT101 and pER8 plasmid, respectively. The PCR products were excised with restriction enzymes and examined in gel electrophoresis (**Supplement 3**). Subsequently, the PCR products were cloned in the pJET1.2/blunt cloning vector (Thermo Scientific). This vector is much easier for cloning and has high positive clone efficiency. In addition, the insert fragment cloned in this vector can be directly checked by sequencing. After DNA sequencing and verifying the correct sequence, the CrGES and ΔplCrGES fragments were ligated into the pTH2-Δ*EcoRI*, pRT101, and pER8 plasmids and subsequently multiplied in *E. coli*. The constructs were examined with restriction enzymes after the plasmids were purified from *E. coli* (**Supplement 4 – 6**).

Transformation of the vectors containing CrGES or ΔplCrGES into *C. roseus* cells was realized via particle bombardment. Initially, two wild-type lines of *C. roseus* cell

suspensions were used for transformation, i.e. the MP183L and the CRPP line. Both cell lines are different in phenotype and metabolites. CRPP is a high producing-TIA cell line, which accumulates among others the secoiridoid precursor loganic acid, and the alkaloids strictosidine, serpentine, tabersonine, and catharanthine. On the other hand, the MP183L line accumulates only the TIA precursors tryptophan and tryptamine, but no secoiridoid precursors nor TIA under the standard growth conditions. Nevertheless, the MP183L line is commonly used for transformation studies in our laboratory as it is easily transformed and giving stable transgenic cell lines. **Figure 2** shows the putative transformed MP183L calli carrying the antibiotic selectable marker, grown on LS medium supplemented with hygromycin. The average numbers of transformed MP183L calli grown per plate were 10 and 26 for pRT101 and pER8 construct, respectively. However, the bombarded CRPP cells did not produce any callus on the selection medium due to unsuccessful transformation. As compared to the fine cells of MP183L, the CRPP cells form aggregates which may affect the transformation efficiency. An additional transient expression study using the GUS assay for evaluation also showed a better transformation efficiency in MP183L compared to the CRPP cell line (**Supplement 7**).

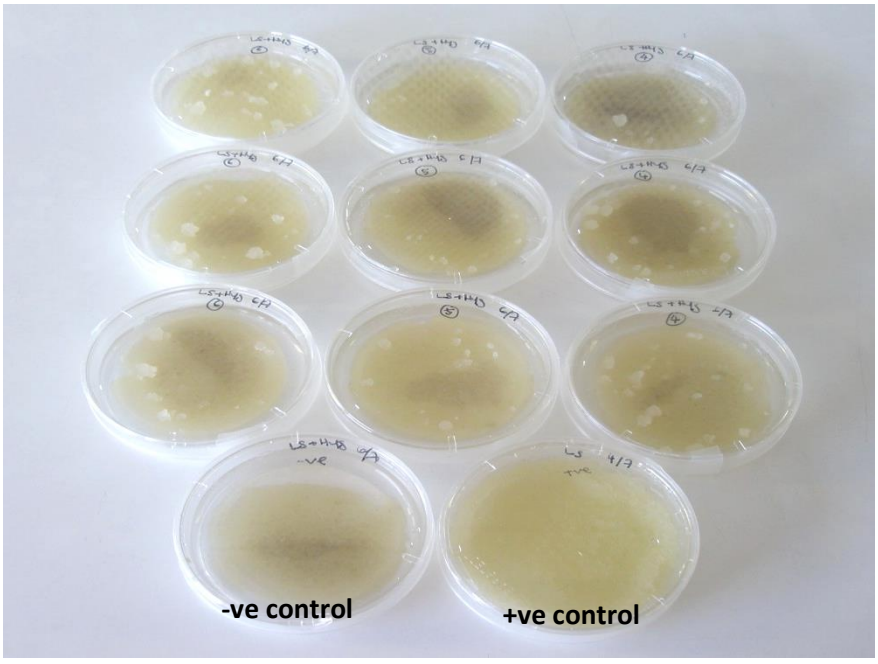

Fig. 2 *Catharanthus roseus* cells bombarded with inducible constructs of CrGES and ΔplCrGES generate putative transformed calli on the culture medium containing hygromycin as selection marker.

Subcellular localization study

To confirm the subcellular localization of CrGES and ΔplCrGES, the transient expression of the construct pTH2-Δ*EcoRI*::CrGES-GFP and pTH2-Δ*EcoRI*::ΔplCrGES-GFP was analyzed in the transformed *C. roseus* cells (MP183L line). The results show that the truncated CrGES without plastidial leader peptide fused with GFP (ΔplCrGES-GFP) was displaying fluorescence in the cytosol (**Fig. 3A – B**). In accordance to Simkin et al. (2013), the full length CrGES-GFP fusion protein signal was located in the plastid stroma and stromules (**Fig. 3C**). To further confirm the subcellular compartments, we co-bombarded the CrGES-GFP and plastid-mCherry marker (Nelson et al. 2007) in the *C. roseus* cells. The results show that the fluorescence signal of CrGES-GFP matched with those of the plastidial marker (**Fig. 3D**), thus confirming its localization in the plastid stroma and stromules. After removing its plastidial leader peptide, the enzyme was expressed in the cytosol.

Gene expression

Expression of CrGES and ΔplCrGES in the putatively transformed *C. roseus* cells was analyzed using Northern blots. **Figure 4A and 5A** show the expression of CrGES and ΔplCrGES in some lines of the *C. roseus* cells transformed with the constitutive or the estradiol-inducible construct, respectively. However, the Northern blot analyses displayed some background noise and tailing possibly caused by the probe or membrane. To verify the results of the Northern blot analyses, reverse transcriptase PCR (RT-PCR) was performed on the same purified RNA. The RT-PCR results reveal the expected sizes of the CrGES and ΔplCrGES fragments with a relatively similar expression level as shown in the Northern Blot (**Fig. 4A – B and 5A – B**). Therefore, these results confirm the overexpression of the CrGES (plastid) and ΔplCrGES (cytosol) in both constitutive and estradiol-induced transgenic *C. roseus* cell suspensions.

Terpenoid indole alkaloid analysis

It was of interest to analyze TIA production in the *C. roseus* cells overexpressing geraniol synthase, particularly to compare the difference between plastidial and cytosolic CrGES overexpression lines. Therefore, several lines of constitutive and inducible transgene *C. roseus* cells were selected and analyzed for the TIA, iridoid and indole precursors using an HPLC-DAD platform.

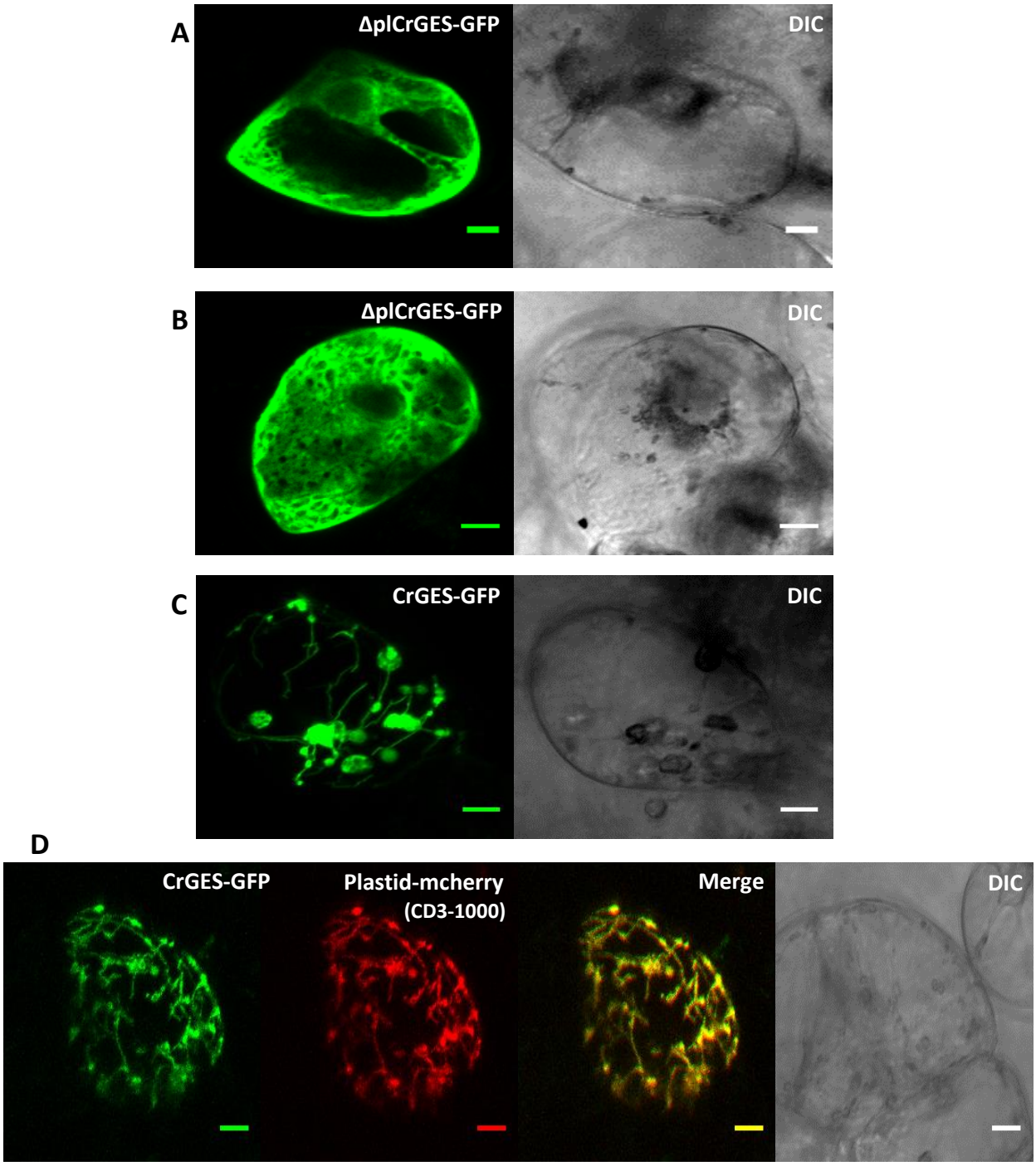

Fig. 3 Subcellular localization of ΔplCrGES-GFP (A, B) and CrGES-GFP (C) in cytosol and plastid/stromules of *Catharanthus roseus* cells, respectively. Co-localization of the two fluorescence signals appeared in yellow when merging the two individual (green/red) color images (D). The cell morphology is observed with differential interference contrast (DIC) microscopy. Bars correspond to 10 µm.

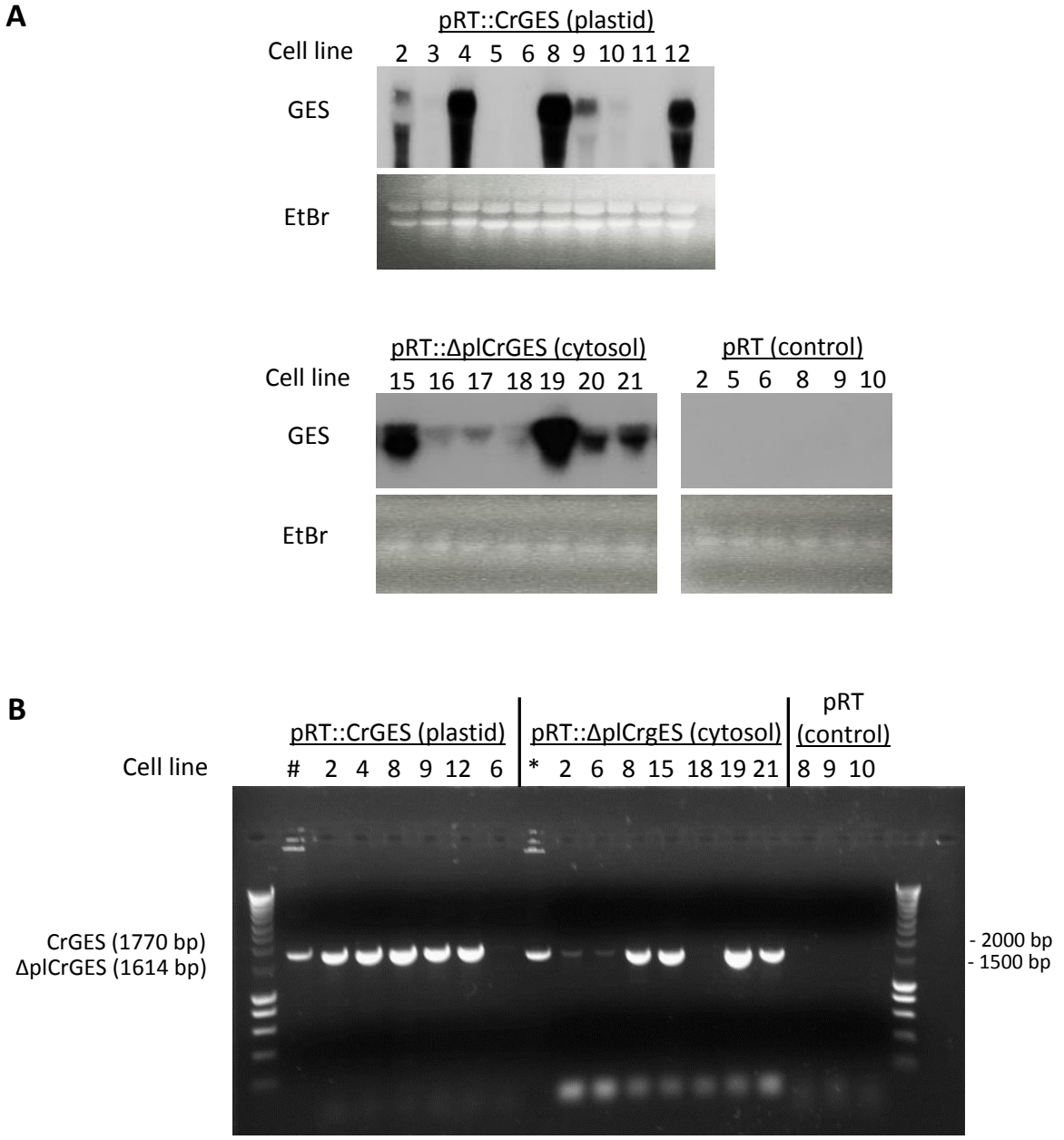

Fig. 4 Northern blot (A) and RT-PCR (B) analysis of independent transgenic *Catharanthus roseus* cell lines constitutively overexpressing CrGES (plastid), and ΔplCrGES (cytosol) versus cells transformed with the corresponding empty vector (control). Analysis of gene expression using Northern blot shows noise and tailing signals and therefore RT-PCR is used to supplement expression data. The ethidium bromide stained gel (EtBr) is shown as a control for RNA loading. # and * in RT-PCR represent the CrGES and ΔplCrGES fragments, respectively, which are used for comparing the sizes of the bands.

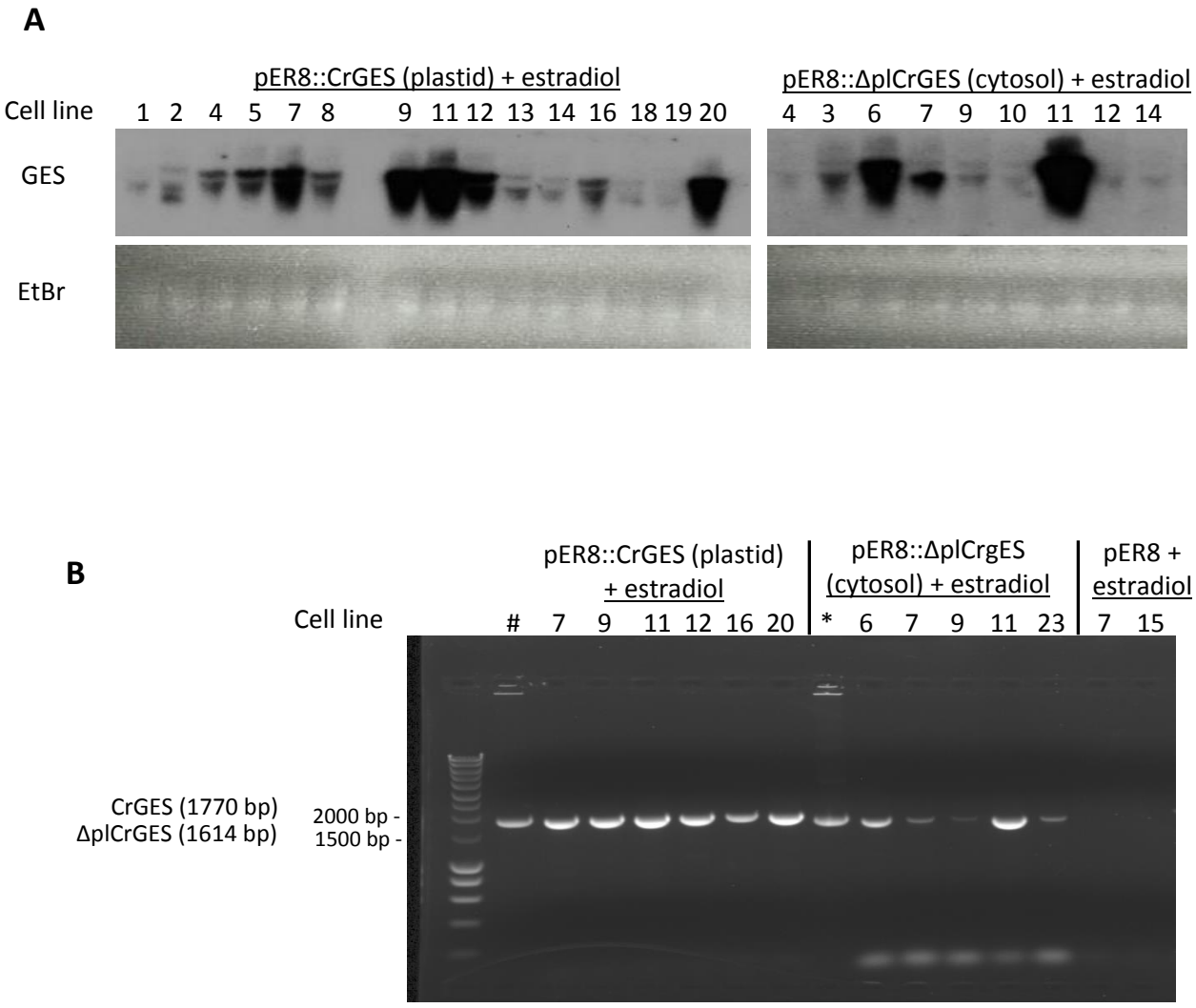

Fig. 5 Northern blot (A) and RT-PCR (B) analysis of independent transgenic *Catharanthus roseus* cell lines overexpressing estradiol-inducible CrGES (plastid), and ΔplCrGES (cytosol) versus control cells transformed with the corresponding empty vector. All samples were fed with estradiol (10 μM) at the fifth day after subculturing and harvested after 24 hours. Analysis of gene expression using Northern blot shows noise and tailing signals and therefore RT-PCR is used to supplement expression data. The ethidium bromide stained gel (EtBr) is shown as a control for RNA loading. # and * in RT-PCR represent CrGES and ΔplCrGES fragments, respectively, which are used for comparing the sizes of the bands.

Terpenoid indole alkaloids or iridoid precursors were not detected in any of these transformed *C. roseus* cell cultures, neither in constitutive nor inducible lines. Similar to the previous analysis of the wild-type *C. roseus* cell-line MP183L, only tryptophan and tryptamine were detected in the cultures. This indicates that geraniol synthase overexpression in the plastid or cytosol of these *C. roseus* cells does not directly enhance fluxes towards iridoid and TIA biosynthesis. Furthermore, in order to build a metabolic bridge between GPP from mevalonate pathway to the iridoids and subsequently induce TIA production, the upstream precursor mevalonic acid (0.5 µM final concentration) was fed to the transgenic *C. roseus* cells constitutively overexpressing ΔplCrGES. However, this did not induce any difference in production levels. A lack of one or more biosynthesis enzymes before or after geraniol synthase could be the reason that the transformed MP183L cell lines did not produce TIA or iridoid precursors. Our previous study showed that the CATL cell line; which derived from MPL183L line, has low gene transcript levels of DXR and G8O (also known as G10H) if compared to the TIA-accumulating CRPP cell line (Saiman et al. 2014). Overexpression of G8O (G10H) in *C. roseus* hairy roots (Wang et al. 2010) and plants (Pan et al. 2012) has increased the levels of several TIAs which indicates that the G8O (G10H) availability is critical for TIA production. It is thus of interest to overexpress CrGES in iridoid- or TIA-accumulating *C. roseus* cell cultures to study the carbon flux in the iridoid or TIA pathway.

The transgenic *C. roseus* cell lines in this study did not accumulate TIA or iridoid precursors, however other metabolic changes may have occurred in the transgenic cells, which is studied by NMR (see below). Nevertheless, to evaluate TIA production in the transformed MP183L line, the transgenic cell lines constitutively overexpressing CrGES or ΔplCrGES, and the control lines containing the empty vector were elicited with jasmonic acid (100 µM). Jasmonate is a defense signaling compound and is often used as elicitor to induce secondary metabolism in plant. Jasmonate elicitation has been reported to induce all known TIA pathway genes including *GES* (Simkin et al. 2013), *G8O* or *G10H* (Collu et al. 2001), *ASα, DXS, TDC, STR,* and *SGD* (van der Fits and Memelink 2000), resulting in increased levels of TIA in *C. roseus* cell suspension (El-Sayed and Verpoorte 2004; Vázquez-Flota et al. 2009). After elicitation, ajmalicine, tabersonine, and a tabersonine-like compound were detected in the cell cultures. Tryptamine levels increased after elicitation as jasmonic acid induces the expression of *TDC*, but no iridoid precursors were detected in the samples. **Figure 6** shows that most of the cell lines accumulated the alkaloids at 48 and 72 hours after

elicitation. The cell line ΔplCrGES #19 accumulated higher levels of alkaloids compared to the other cell lines. However, the alkaloid production levels in the control lines were quite variable upon elicitation; whereas one empty vector control line did not accumulate alkaloids after elicitation, control line #8 produced alkaloids at a comparable level to that produced by the CrGES or ΔplCrGES lines. In this perspective only line ΔplCrGES #19 revealed higher TIA production than controls. Therefore, we conclude that jasmonic acid elicitation did not cause a clear difference in TIA level between the geraniol synthase overexpressing cells and the controls as the elicitation effect seems to vary too much among the individual cell lines.

Geraniol analysis

No geraniol peak was detected by GC in the plastidial CrGES or cytosolic ΔplCrGES overexpressing cells, which might be due to 1) the lack of carbon-five precursors because of limiting activity of upstream biosynthesis enzymes, 2) competing pathways acting on GPP, e.g. leading to FPP and sterols or to GGPP and carotenoids, or 3) the conversion of geraniol to glycosylated derivatives. Dong et al. (2013) reported that tobacco plants overexpressing geraniol synthase from *Valeriana officinalis* (VoGES) predominantly accumulated geraniol glycoside. Therefore, further work needs to be done to either analyze the glycosylated geraniol compounds in the transformed MP183L lines or treat the cells with glycosidase to release geraniol and geraniol related compounds.

Expression of VoGES to the cytosolic mevalonate compartment resulted in 30% lower geraniol glycoside than the plastidial targeted VoGES (Dong et al. 2013). This indicates a smaller pool of GPP in the mevalonate pathway compared to the MEP pathway. It may be interesting to overexpress both GPPS and GES in the mevalonate pathway of *C. roseus* as Wu et al. (2006) showed that the co-expression of limonene synthase (LS) and GPPS in the cytosolic mevalonate pathway increased production of limonene 6-fold compared to the single overexpression of LS. However, the targeted subcellular compartment for GPPS expression in the mevalonate pathway needs to be evaluated since the IPP isomerase (Sapir-Mir et al. 2008) and FPPS (Thabet et al. 2011), which were generally regarded as cytosolic enzymes, were recently reported to be localized in peroxisomes. Nevertheless, Thabet et al. (2011) did not exclude the possibility that a certain proportion of the FPPS is also localized in the cytosol. Furthermore, down-regulation of FPPS may be an interesting approach since a mutated FPPS yeast strain overexpressing *Ocimum basilicum* geraniol synthase (ObGES) accumulated geraniol produced from the available GPP pool (Fischer et al. 2011).

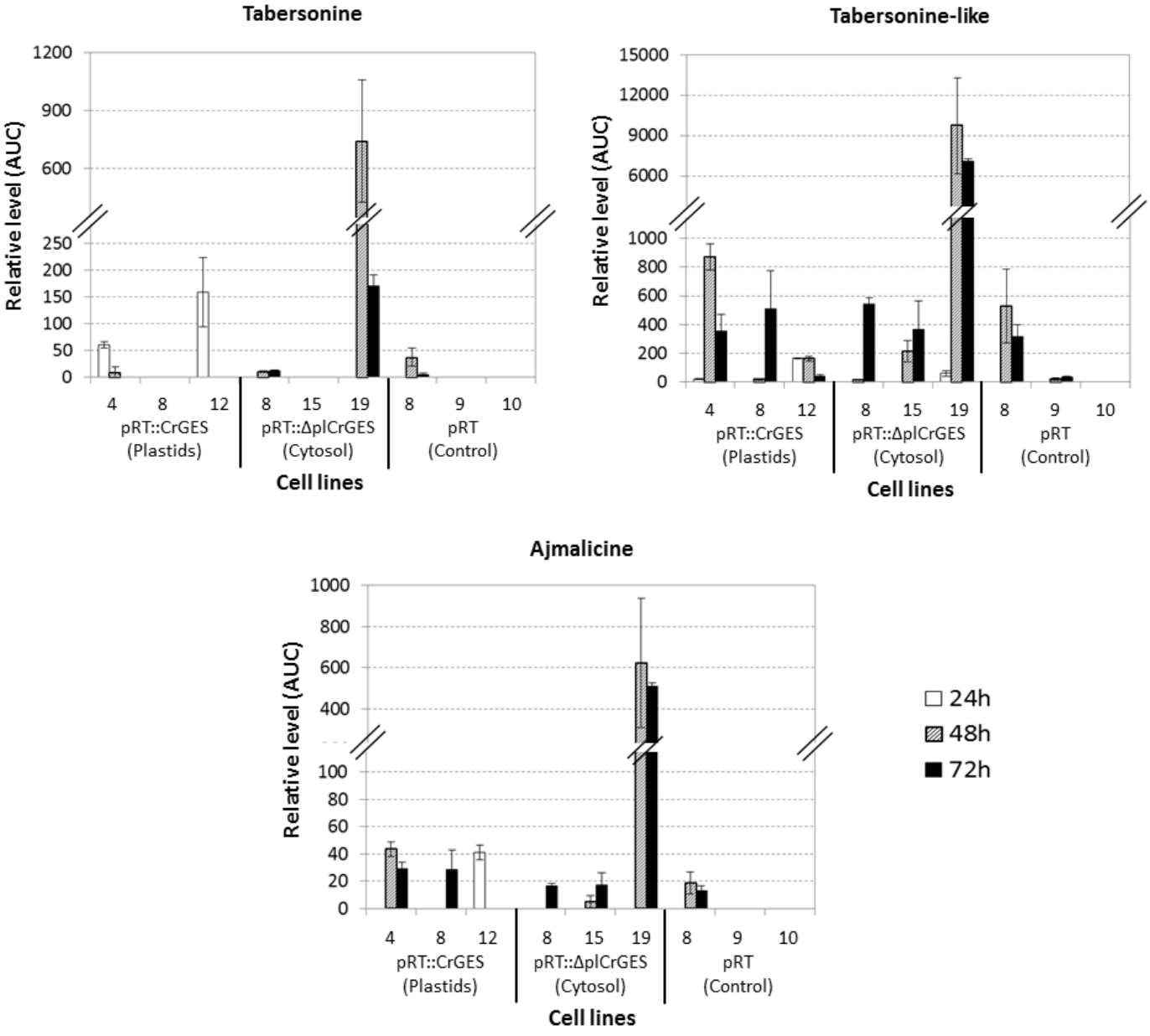

Fig. 6 Relative levels of terpenoid indole alkaloids (ajmalicine, tabersonine, and tabersonine-like compound) detected by HPLC-Diode Array Detector (DAD) in jasmonate-elicited *Catharanthus roseus* cell suspensions constitutively overexpressing CrGES (plastids; cell line 4, 8, and 12), ΔplCrGES (cytosol; cell line 8, 15, and 19) and empty vector pRT (control; cell line 8, 9, and 10). All samples were elicited with jasmonic acid (100 μM) at the fifth day after subculturing and harvested at 24, 48, and 72 hours after elicitation. Results are the mean of two replicates; error bars indicate the two values. AUC is area under the curve value.

NMR-based metabolomics and multivariate data analysis

In order to further investigate the metabolic differences in the non-elicited transformed *C. roseus* cells, NMR-based metabolomics was conducted and the ^{1}H-NMR data were subjected to multivariate data analysis. Principal component analysis (PCA) which is an unsupervised clustering method was initially performed to discriminate the samples. **Figure 7A** shows a PCA score plot of constitutive CrGES (three cell lines), ΔplCrGES (three cell lines), and control-empty vector (five cell lines). PC1 and PC2 explained 45% and 33% of variation, respectively. However, PCA did not clearly separate the different cell lines, which means that the variation between the groups is smaller than that within the individual cell lines. Therefore, partial least squares-discriminant analysis (PLS-DA) was applied to the same ^{1}H-NMR data to specifically examine the metabolite differences between the different construct lines.

Partial least squares-discriminant analysis (PLS-DA) is a supervised multivariate data analysis that uses information in another matrix. In addition to the X-matrix of NMR data, three groups (CrGES, ΔplCrGES, and empty vector) were assigned for the Y-matrix in PLS-DA. **Figure 7B** shows the PLS-DA score plot of the samples, in which the separation between the groups has considerably improved. The PLS-DA model was validated by the permutation method through 20 applications, in which the Q2 values of permuted Y vectors to the left were lower than the original ones to the right and the regression of Q2 lines intersect vertical axis at below zero (**Fig. 7C**). To get a better sample separation from the validated PLS-DA model, orthogonal projection to latent structures-discriminant analysis (OPLS-DA) was applied to the same data.

In OPLS-DA, a single component is used as a predictor for the class/group, while the other components describe the variation orthogonal to the first predictive component (Westerhuis et al. 2010). **Figure 7D** shows a clear separation between three groups (CrGES, ΔplCrGES, and empty vector) in the OPLS-DA score plot. All CrGES overexpressing cell lines were grouped in the positive area of component 1 and the negative area of component 2, while ΔplCrGES overexpressing cell lines were in negative areas of component 1 and 2, and the control cell lines (empty vector) were grouped in negative and positive area of component 1 and 2, respectively.

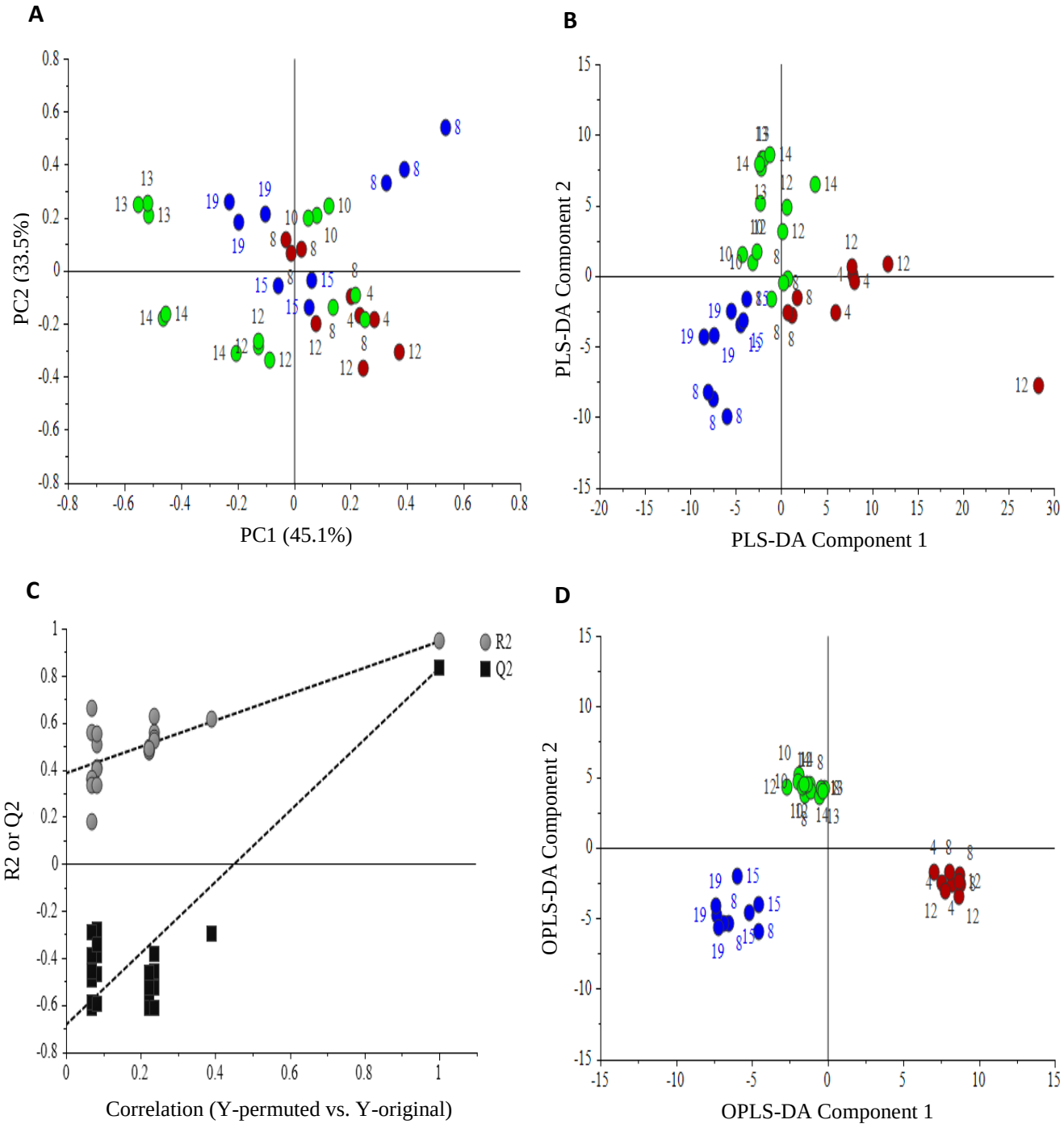

Fig. 7 Multivariate data analyses of *Catharanthus roseus* cells constitutively overexpressing CrGES (red color), ΔplCrGES (blue color), and the control *C. roseus* cells transformed with empty vector (green color). Principal component analysis (PCA) score plot (A), partial least squares-discriminant analysis (PLS-DA) score plot (B), and orthogonal projection to latent structures-discriminant analysis (OPLS-DA) score plot (D). The numbers in the score plots represent the cell-line label. Validation of PLS-DA by permutation test (C). R2 is a measurement of the model's goodness of fit. Q2 is a measurement of the predictive ability of the model.

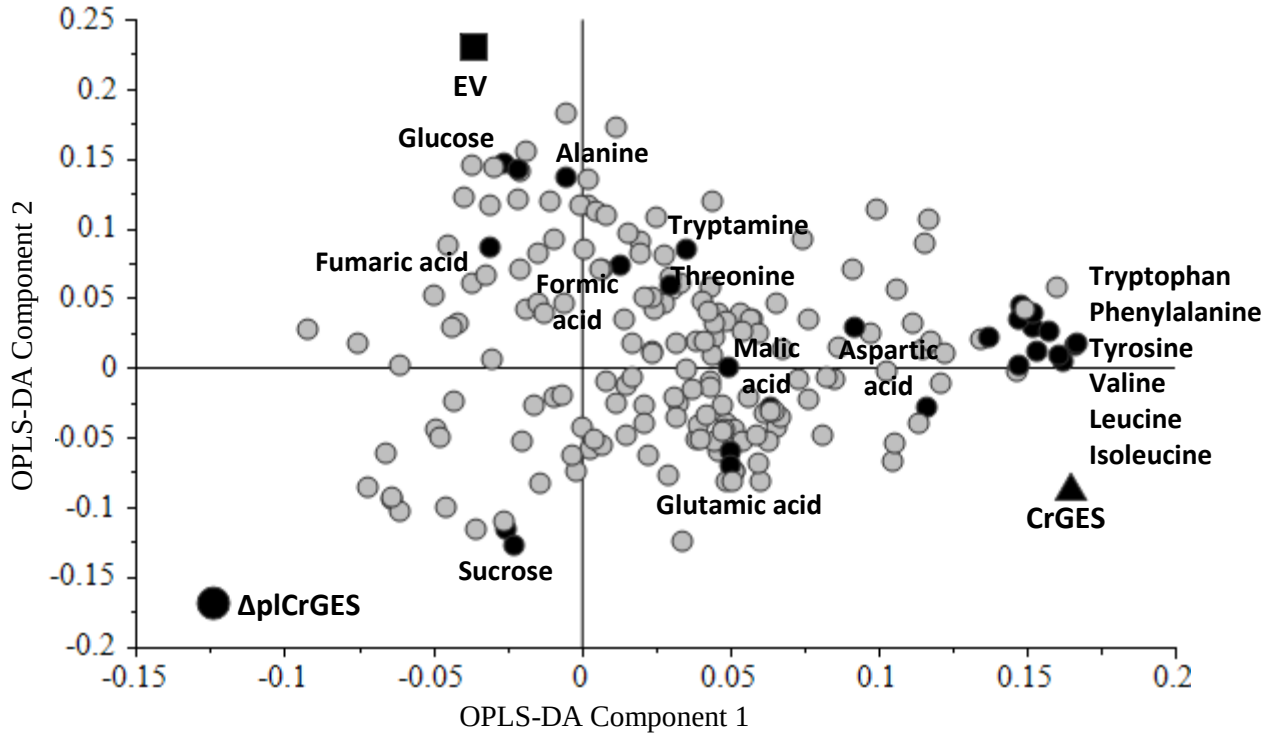

Fig. 8 Loading plot of orthogonal projection to latent structures-discriminant analysis (OPLS-DA) of *Catharanthus roseus* cells constitutively overexpressing CrGES, ΔplCrGES, and the control *C. roseus* cells transformed with empty vector (EV). Variables X (metabolite signals) located closely to variable Y (CrGES, ΔplCrGES, EV) contributes to the separation of the samples.

The NMR signals of some metabolites detected in the samples were assigned (**Table 1)**. The loading plot (**Fig. 8**) reveals which metabolites contribute to the separation of the groups. The result suggests that tryptophan, phenylalanine, tyrosine, valine, leucine, isoleucine, aspartic acid, malic acid, and glutamic acid are the metabolites that strongly influence the separation of the constitutive CrGES overexpressing cell lines. In the constitutive ΔplCrGES overexpressing cell lines, sucrose was associated to the separation of this group. Glucose, alanine, fumaric acid, and formic acid were detected close to the component area where the control lines were located, suggesting the influence of these metabolites for defining the control cell lines. **Figure 9** compares aromatic region of ^{1}H-NMR spectra of the different cell lines in the constitutive CrGES, ΔplCrGES, and the empty vector transformed *C. roseus* cell cultures.

In order to confirm that the metabolites are statistically significant for the separation of the groups, an ANOVA test ($P < 0.05$) was performed by comparing the mean value of the metabolites between the groups of observation; each group consists of three to five different

Table 1. ^{1}H chemical shift (δ in ppm) and coupling constants (J in Hz) of some metabolites detected in the transgenic and control cell cultures of *Catharanthus roseus. s* = singlet, *d* = doublet, *dd* = double doublet, *t* = triplet, *m* = multiplet

Compounds	**Chemical shift (ppm) and coupling constant (Hz)**
Leucine	δ 0.97 (*d*, J = 6.8); δ 0.99 (*d*, J = 6.8)
Isoleucine	δ 0.96 (*t*, J = 7.5); δ 1.03 (*d*, J = 7.0)
Valine	δ 1.01 (*d*, J = 7.0); δ 1.06 (*d*, J = 7.0)
Threonine	δ 1.34 (*d*, J = 6.6)
Alanine	δ 1.49 (*d*, J = 7.2)
Glutamic acid	δ 2.04 (*m*); δ 2.12 (*m*); δ 2.39 (*m*)
Glutamine	δ 2.13 (*m*); δ 2.46 (*m*)
Malic acid	δ 2.68 (*dd*, J = 15.4, 3.3); δ 4.28 (*dd*, J = 9.5, 3.2)
Aspartic acid	δ 2.82 (*dd*, J = 17.0, 8.0); δ 2.95 (*dd*, J = 16.8, 4.0); δ 3.92 (*dd*, J = 8.4, 4.0)
Sucrose	δ 4.18 (*d*, J = 8.6); δ 5.41 (*d*, J = 3.8)
Glucose	δ 4.58 (*d*, J = 8.0, β-form); δ 5.19 (*d*, J = 3.8, α-form)
Fumaric acid	δ 6.52 (*s*)
Tyrosine	δ 6.85 (*d*, J = 8.5) δ 7.19 (*d*, J = 8.5)
Tryptophan	δ 7.14 (*t*, J = 7.5); δ 7.22 (*t*, J = 7.5); δ 7.29 (*s*); δ 7.48 (*d*, J = 8.0); δ 7.73 (*d*, J = 8.0)
Tryptamine	δ 7.14 (*t*, J = 7.5); δ 7.22 (*t*, J = 7.5); δ 7.28 (*s*); δ 7.48 (*d*, J = 8.0); δ 7.65 (*d*, J = 8.0)
Phenylalanine	δ 7.36 (*m*)
Formic acid	δ 8.48 (*s*)

cell lines and each cell line consists of three biological replicates. Of the identified metabolites, tryptophan, phenylalanine, tyrosine, valine, leucine, and isoleucine were significantly different ($P < 0.05$) between the groups (**Fig. 10**). It was interesting to note that the phenylalanine level was higher (about twofold) in the constitutive CrGES overexpressing cells, but lower (twofold) in the ΔplCrGES overexpressing cells, compared to the control (empty vector). The higher and lower level pattern was also observed for tyrosine, valine, and leucine. Isoleucine level in the constitutive ΔplCrGES overexpressing cell cultures was not significantly different from the control, but it was significantly higher in the constitutive CrGES overexpressing cells. For tryptophan, neither CrGES nor ΔplCrGES overexpressing cell lines were statistically different compared to the control lines but the CrGES overexpressing cells have 2.5-fold more tryptophan than that in ΔplCrGES overexpressing cells. No iridoids and TIA signals were detected in NMR, similar to the HPLC analysis. The only TIA precursor present in the samples, tryptamine was not significantly different between the groups. These results indicate that constitutive overexpression of CrGES in the plastid

and ΔplCrGES in the cytosol influenced the levels of some primary metabolites (including phenylalanine, tyrosine, and tryptophan, which derive from the shikimate pathway) while each displayed contrasting effects. There is no information on the involvement of geraniol synthase in the shikimate/phenylpropanoid pathway but there is a report by Sung et al. (2011) suggesting that the cytochrome P450 enzyme which converts geraniol to 10-oxogeranial, i.e. G8O (G10H), is not only involved in terpenoid pathway but also plays an important role in flavonoid/phenylpropanoid pathway. Further study should carry out for a better understanding of the total metabolic network.

The inducible CrGES and ΔplCrGES transgene *C. roseus* cell suspensions were also analyzed by NMR and multivariate data analysis. In the case of the inducible lines, PLS was used as supervised method and two Y-matrices were assigned, i.e. the construct groups (CrGES, ΔplCrGES, and control-empty vector) and the treatment (estradiol vs. DMSO).

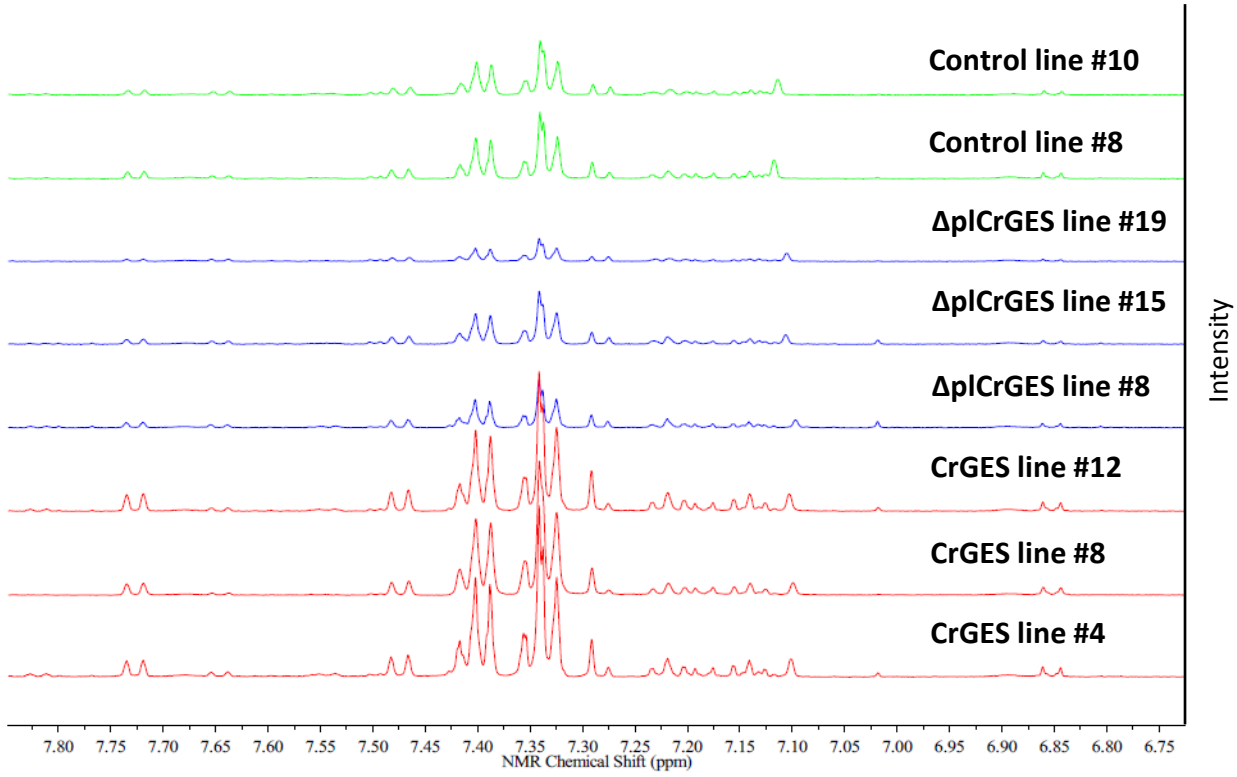

Fig. 9 Comparison of ^{1}H-NMR spectra of aromatic region of two *Catharanthus roseus* control lines (transformed with empty vector) versus different cell lines constitutively expressing CrGES and ΔplCrGES.

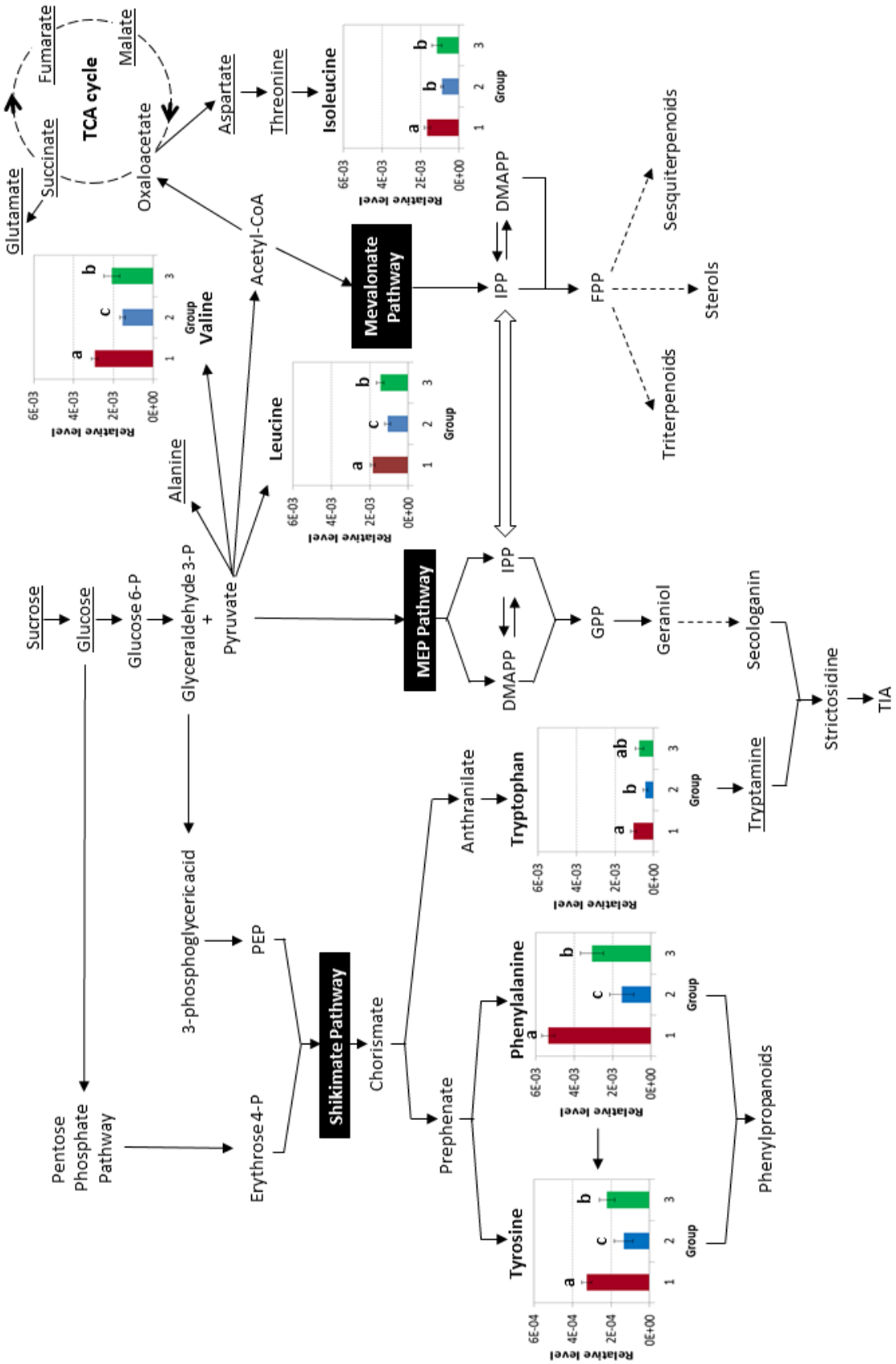

Fig. 10 Schematic representation of metabolic differences between *Catharanthus roseus* cell lines overexpressing CrGES in the plastids (1), ΔplCrGES in cytosol (2), and empty vector/control (3). Relative level of metabolites is the mean area of ^{1}H-NMR resonance peak associated to that metabolite. Each group consists of three to five different cell lines and each cell line is represented by three biological replicates. Means ± SD with different letters are significantly different ($P < 0.05$) using ANOVA followed by Duncan's Multiple Range Test (DMRT). Underlined metabolites are not significantly different in levels.

However, neither PCA nor PLS displayed separation between the lines induced with 10 µM estradiol or control (DMSO). No separation between the induced and control cell lines could be due to the observation time applied on the samples, i.e. cells were harvested 24 hours after treatment which may have been too short to develop a difference in metabolic processes between the control and induced cell lines.

Conclusion

In this study, we describe the development of *C. roseus* cell lines overexpressing geraniol synthase in the plastid (CrGES) or the cytosol (ΔplCrGES). The study confirmed the expression of CrGES and ΔplCrGES in both subcellular compartments. However, geraniol was not detected in the transformed cells, or present below the detection limit. Further analyses are needed to reveal if the GES product is formed and subsequently derivatized to glycosylated geraniol products. No accumulation of TIA or iridoid pathway precursors was detected in the *C. roseus* cells of the line MP183L after overexpressing geraniol synthase, whereas jasmonate acid elicitation did not cause clear differences in TIA production between overexpression and control cultures. NMR-based metabolomics combined with multivariate data analysis revealed some primary metabolites associated to the separation of the constitutive CrGES, ΔplCrGES *C. roseus* cells and controls in PLS-DA/OPLS-DA analysis. In contrast to a higher level of several metabolites of which some are associated to shikimate pathway in the constitutive CrGES overexpressing *C. roseus* cells, a lower level of these metabolites was detected in the ΔplCrGES overexpressing cultures compared to the control, thus suggesting different metabolic effects related to the subcellular compartmentation of geraniol synthase.

Acknowledgements

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References

Canel C, Lopes-Cardoso MI, Whitmer S, van der Fits L, Pasquali G, van der Heijden R, Hoge JHC, Verpoorte R (1998) Effects of overexpression of strictosidine synthase and tryptophan decarboxylase on alkaloid production by cell cultures of *Catharanthus roseus*. Planta 205: 414–419

Contin A, van der Heijden R, Lefeber AW, Verpoorte R (1998) The iridoid glucoside secologanin is derived from the novel triose phosphate/pyruvate pathway in a *Catharanthus roseus* cell culture. FEBS Lett 434: 413–416

Dong L, Miettinen K, Goedbloed M, Verstappen FW, Voster A, Jongsma MA, Memelink J, van der Krol S, Bouwmeester HJ (2013) Characterization of two geraniol synthases from *Valeriana officinalis* and *Lippia dulcis*: similar activity but difference in subcellular localization. Metab Eng 20: 198–211

Fischer MJ, Meyer S, Claudel P, Bergdoll M, Karst F (2011) Metabolic engineering of monoterpene synthesis in yeast. Biotechnol Bioeng 108: 1883–1892

Ishige F, Takaichi M, Foster R, Chua NH, Oeda K (1999) A G-box motif (GCCACGTGCC) tetramer confers high-level constitutive expression in dicot and monocot plants. Plant J 18: 443–448

Jaggi M, Kumar S, Sinha AK (2011) Overexpression of an apoplastic peroxidase gene *CrPrx* in transgenic hairy root lines of *Catharanthus roseus*. Appl Microbiol Biotechnol 90: 1005–1016

Linsmaier EM, Skoog F (1965) Organic growth factor requirements of tobacco tissue cultures. Physiol Plant 18: 100–127

Liu DH, Ren WW, Cui LJ, Zhang LD, Sun XF, Tang KX (2011) Enhanced accumulation of catharanthine and vindoline in *Catharanthus roseus* hairy roots by overexpression of transcriptional factor ORCA2. Afr J Biotechnol 10: 3260–3268

Magnotta M, Murata J, Chen J, De Luca V (2007) Expression of deacetylvindoline-4-*O*-acetyltransferase in *Catharanthus roseus* hairy roots. Phytochemistry 68: 1922–1931

Martin D, Piulachs MD, Cunillera N, Ferrer A, Bellés X (2007) Mitochondrial targeting of farnesyl diphosphate synthase is a widespread phenomenon in eukaryotes. Biochim Biophys Acta 1773: 419–26

Memelink J, Swords KMM, Staehelin LA, Hoge JHC (1994) Southern, Northern and Western blot analysis. In: Gelvin SB, Schilperoot RA (eds.) Plant Molecular Biology Manual. Kluwer Academic Publishers, Dordrecht, pp. 273–295

Moreno PRH, van der Heijden R, Verpoorte R (1995) Cell and tissue cultures of *Catharanthus roseus*: a literature survey. Plant Cell Tiss Organ Cult 42: 1–25

Nelson BK, Cai X, Nebenführ A (2007) A multicolored set of in vivo organelle markers for co-localization studies in *Arabidopsis* and other plants. Plant J 51: 1126–1136

Niwa Y (2003) A synthetic green fluorescent protein gene for plant biotechnology. Plant Biotechnol 20: 1–11

Pan Q, Wang Q, Yuan F, Xing S, Zhao J, Choi YH, Verpoorte R, Tian Y, Wang G, Tang K (2012) Overexpression of ORCA3 and G10H in *Catharanthus roseus* plants regulated alkaloid

biosynthesis and metabolism revealed by NMR-metabolomics. PLoS ONE 7(8):e43038. doi:10.1371/journal.pone.0043038

Peebles CA, Hughes EH, Shanks JV, San KY (2009) Transcriptional response of the terpenoid indole alkaloid pathway to the overexpression of ORCA3 along with jasmonic acid elicitation of *Catharanthus roseus* hairy roots over time. Metab Eng 11: 76–86

Peebles CAM, Sander GW, Hughes EH, Peacock R, Shanks JV, San KY (2010) The expression of 1-deoxy-D-xylulose synthase and geraniol 10-hydroxylase or anthranilate synthase increases terpenoid indole alkaloid accumulation in *Catharanthus roseus* hairy roots. Metab Eng 13: 234–40

Pomahačová BDJ, Dusková J, Yazaki K, Roytrakul S, Verpoorte R (2009) Improved accumulation of ajmalicine and tetrahydroalstonine in *Catharanthus* cells expressing an ABC transporter. J Plant Physiol 166: 1405–1412.

Rohmer M (1999) The discovery of a mevalonate-independent pathway for isoprenoid biosynthesis in bacteria, algae and higher plants. Nat Prod Rep 16:565–574

Saiman MZ, Mustafa NR, Pomahačová B, Verberne M, Verpoorte R, Choi YH, Schulte AE (2014) Analysis of metabolites in the terpenoid pathway of *Catharanthus roseus* cell suspensions. Plant Cell Tiss Organ Cult 117: 225–239

Sapir-Mir M, Mett A, Belausov E, Tal-Meshulam S, Frydman A, Gidoni D, Eyal Y (2008) Peroxisomal localization of Arabidopsis isopentenyl diphosphate isomerases suggests that part of the plant isoprenoid mevalonic acid pathway is compartmentalized to peroxisomes. Plant Physiol 148: 1219–1228

Simkin AJ, Guirimand G, Papon N, Courdavault V, Thabet I, Ginis O, Bouzid S, Giglioli-Guivarc'h N, Clastre M (2011) Peroxisomal localisation of the final steps of the mevalonic acid pathway in planta. Planta 234: 903–914

Simkin AJ, Miettinen K, Claudel P, Burlat V, Guirimand G, Courdavault V, Papon N, Meyer S, Godet S, St-Pierre B, Giglioli-Guivarc'h N, Fischer MJ, Memelink J, Clastre M (2013) Characterization of the plastidial geraniol synthase from Madagascar periwinkle which initiates the monoterpenoid branch of the alkaloid pathway in internal phloem associated parenchyma. Phytochemistry 85: 36–43

Sung PH, Fong-Chin Huang FC, Do YY, Pung-Ling Huang PL (2011) Functional expression of geraniol 10-hydroxylase reveals its dual function in the biosynthesis of terpenoid and phenylpropanoid. J Agric Food Chem 59: 4637–4643

Thabet I, Guirimand G, Courdavault V, Papon N, Godet S, Dutilleu C, Bouzid S, Giglioli-Guivarc'h N, Clastre M, Simkin AJ (2011) The subcellular localization of periwinkle farnesyl diphosphate synthase provides insight into the role of peroxisome in isoprenoid biosynthesis. J Plant Physiol 168: 2110–2116

Töpfer R, Matzeit V, Gronenborn B, Schell J, Steinbiss HH (1987) A set of plant expression vectors for transcriptional and translational fusions. Nucleic Acids Res 15: 5890

van der Fits L, Memelink J (1997) Comparison of the activities of CaMV 35S and FMV 34S promoter derivatives in *Catharanthus roseus* cells transiently and stably transformed by particle bombardment. Plant Mol Biol 33: 943–946

van der Fits L, Memelink J (2000) ORCA3, a jasmonate-responsive transcriptional regulator of plant primary and secondary metabolism. Science 289: 295–297

van der Heijden R, Verpoorte R, Ten Hoopen HJG (1989) Cell and tissue cultures of *Catharanthus roseus* (L.) G. Don: a literature survey. Plant Cell Tiss Organ Cult 18: 231–280

van der Heijden R, Jacobs DI, Snoeijer W, Hallard D, Verpoorte R (2004) The *Catharanthus* alkaloids: pharmacognosy and biotechnology. Curr Med Chem 11: 607–628

Verpoorte R, Contin A, Memelink J (2002) Biotechnology for the production of plant secondary metabolites. Phytochem Rev 1: 13–25

Verpoorte R, van der Heijden R, Memelink J (2000) Engineering the plant cell factory for the secondary metabolite production. Transgenic Res 9: 323-343

Wang CT, Liu H, Gao XS, Zhang HX (2010) Overexpression of G10H and ORCA3 in the hairy roots of *Catharanthus roseus* improves catharanthine production. Plant Cell Rep 29: 887–894

Westerhuis JA, van Velzen EJJ, Hoefsloot HCJ, Smilde AK (2010) Multivariate paired data analysis: multilevel PLSDA versus OPLSDA. Metabolomics 6: 119–128

Whitmer S, van der Heijden R, Verpoorte R (2002) Effect of precursor feeding on alkaloid accumulation by a strictosidine synthase over-expressing transgenic cell line S1 of *Catharanthus roseus*. Plant Cell Tissue Organ Cult 69:85–93

Wu S, Schalk M, Clark A, Miles RB, Coates R, Chappell J (2006) Redirection of cytosolic or plastidic isoprenoid precursors elevates terpene production in plants. Nat Biotechnol 24: 1441– 1447

Zhao J, Verpoorte R (2007) Manipulating indole alkaloid production by *Catharanthus roseus* cell cultures in bioreactors: from biochemical processing to metabolic engineering. Phytochem Rev 6: 435–457

Zuo J, Niu QW, Chua NH (2000) An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. Plant J 24: 265–273

Supplement 1 The full-length cDNA of *Catharanthus roseus* geraniol synthase (CrGES) localized in the plastid (Genbank ID: JN882024). Its plastidial leader peptide was predicted to be at least 43 amino acids in length (Simkin et al. 2013). The underlined sequence indicates the first 156 bp (52 amino acids; until the first ATG-methionine following the leader) which were deleted to make the truncated version of ΔplCrGES in this study.

ATGGCAGCCACAATTAGTAACCTTTCTTTCTTAGCAAAATCTAGGGCACTTTCAAGGCCTTCTTCTTCTTCACTT
TCATGGCTAGAAAGGCCTAAAACTTCATCGACTATTTGCATGTCTATGCCATCATCTTCATCATCATCATCATCT
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TCACTTTGTTTTCGATTGCTTCGACACAATGGGCATAAGATCAATCCTGATATATTCCAAAAATTCATGGACAAC
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GCCAATGGAGAAGAAATATTGATGGAAGCCCAAGAATTCACCAAAACTCACCTGAAAAACTCATTGCCAGCCATG
GCACCATCTCTTTCTAAGAAGGTTTCTCAAGCTTTAGAGCAACCAAGACATAGAAGAATGTTGAGGTTAGAAGCT
AGAAGATTTATTGAAGAATATGGTGCTGAAAATGACCATAATCCAGACCTTCTTGAGCTTGCAAAATTGGATTAT
AACAAAGTCCAATCTCTACACCAAATGGAATTGTCTGAGATAACAAGGTGGTGGAAACAATTAGGGCTTGTGGAT
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TCAGGTTGCAGAATTGAGCTTGCAAAAACCATAGCCATTTTGCTTGTCATTGATGATATCTTTGATACTCATGGT
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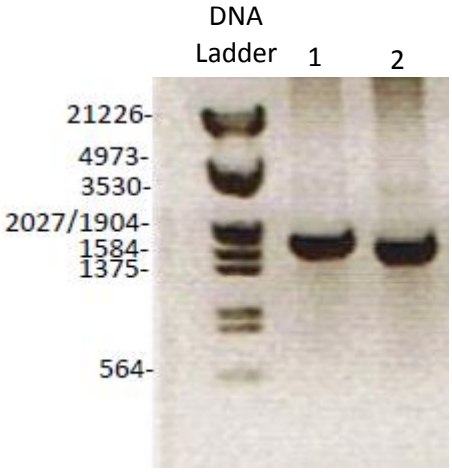
GTCCATTTGTTCTTTCTAATAGGGCAAGGTGTCACTGAAGATAATGTGAAATTACTGATTAAACCCTATCCAAAG
CTCTTTTCCTCCTCAGGAAGAATCCTTCGCCTTTGGGATGATTTGGGAACTGCAAAGGAGGAACAAGAAAGAGGA
GACTTGGCATCAAGCATACAATTGTTTATGAGAGAGAAAGAGATAAAATCAGAAGAAGAAGGAAGGAAAGGAATA
TTGGAAATTATAGAGAATTTATGGAAAGAATTGAATGGAGAATTAGTTTATAGAGAAGAAATGCCTCTTGCAATA
ATCAAGACAGCATTCAACATGGCAAGAGCTTCCCAAGTTGTGTATCAACATGAAGAAGACACCTATTTTTCAAGT
GTAGATAATTATGTAAAAGCTTTGTTTTTTACACCTTGTTTTTAA

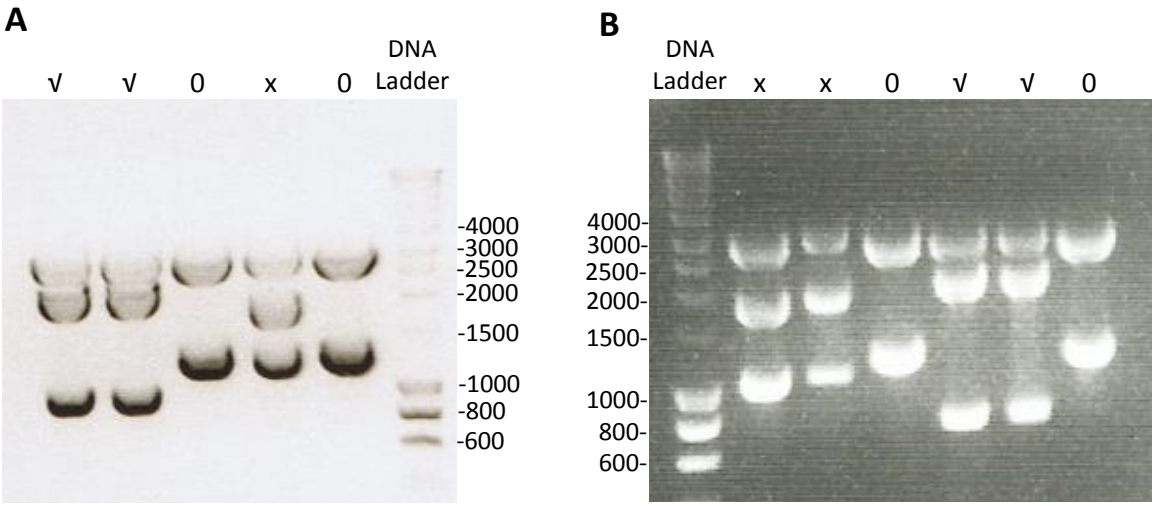
Supplement 2 The primer sequences used for designing full-length cDNA of *Catharanthus roseus* geraniol synthase (CrGES) or a truncated version without its plastidial leader peptide (ΔplCrGES). Construct no. 1 and 2 are the inserts for constitutive and inducible expression. Construct no. 3 and 4 are the inserts for transient expression.

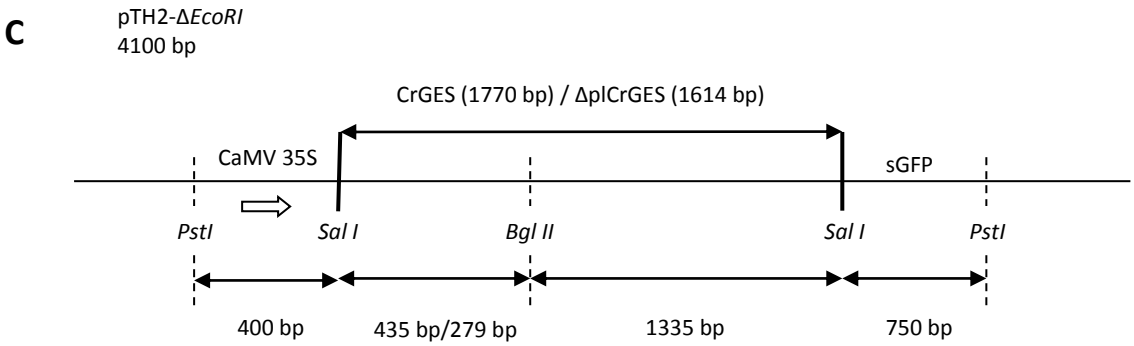
No	Constructs	Primer sequences	
		Forward	Reverse
1.	*SalI*-CrGES-*XbaI*	5'-GTCGACAAAATGGCAGCCACAATTAGTAACC-3'	5'-TCTAGATTAAAAACAAGGTGTAAAAAACAAAGC-3'
2.	*SalI*-ΔplCrGES-*XbaI*	5'-GTCGACAAAATGTCTCTGCCTTTGGCAACT-3'	5'-TCTAGATTAAAAACAAGGTGTAAAAAACAAAGC-3'
3.	*SalI*-CrGES-*SalI*	5'-GTCGACAAAATGGCAGCCACAATTAGTAACC-3'	5'-GTCGACAAAACAAGGTGTAAAAAACAAAGC-3'
4.	*SalI*-ΔplCrGES-*SalI*	5'-GTCGACAAAATGTCTCTGCCTTTGGCAACT-3'	5'-GTCGACAAAACAAGGTGTAAAAAACAAAGC-3'

Supplement 3 The PCR products cut with restriction enzymes *SalI* and *XbaI* examined by gel electrophoresis. 1) full-length fragment of *Catharanthus roseus* geraniol synthase (CrGES; about 1.8 kb). 2) fragment encoding truncated CrGES without plastidial leader peptide (ΔplCrGES; about 1.6 kb).

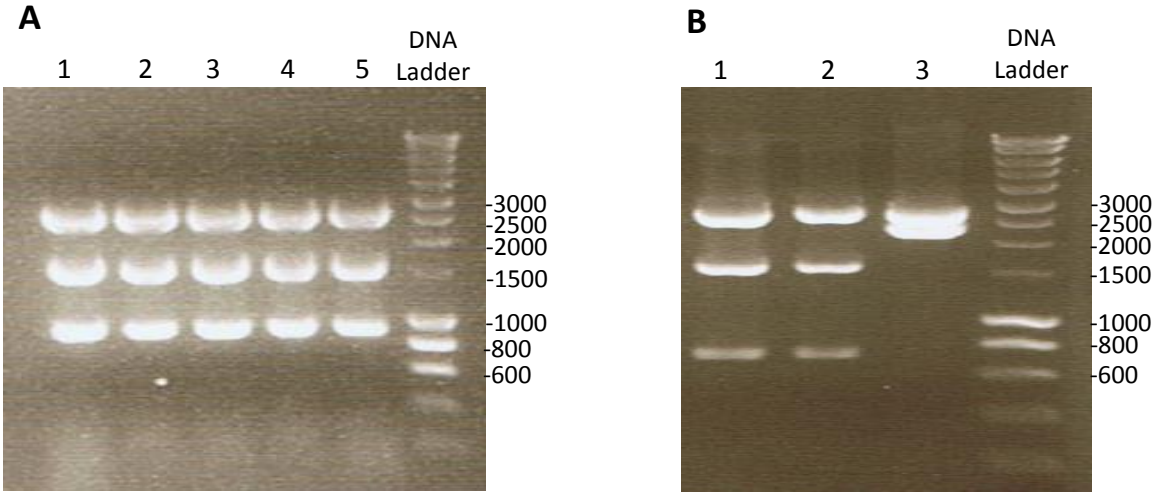


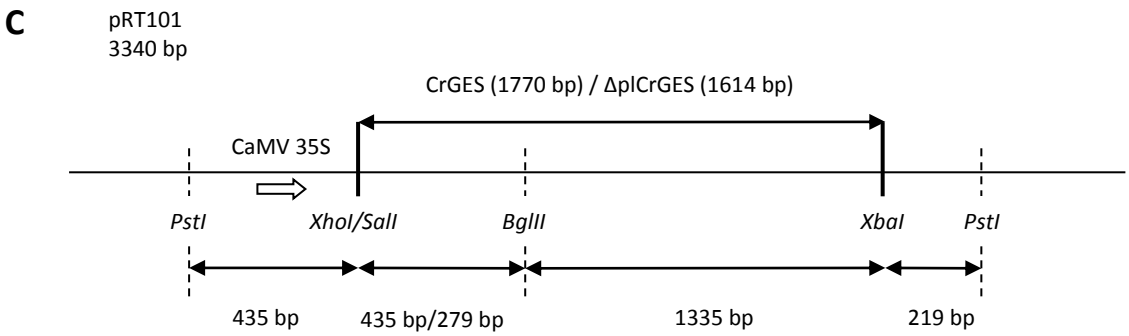
Supplement 4 Constructs of pTH2-Δ*EcoRI*::CrGES-GFP (A) and pTH2-Δ*EcoRI*::Δpl CrGES-GFP (B) excised with *BglII/PstI* give expected sizes of DNA bands as represented in the construct scheme (C). Correct construct (√), empty vector (0), and reverse insert (x).



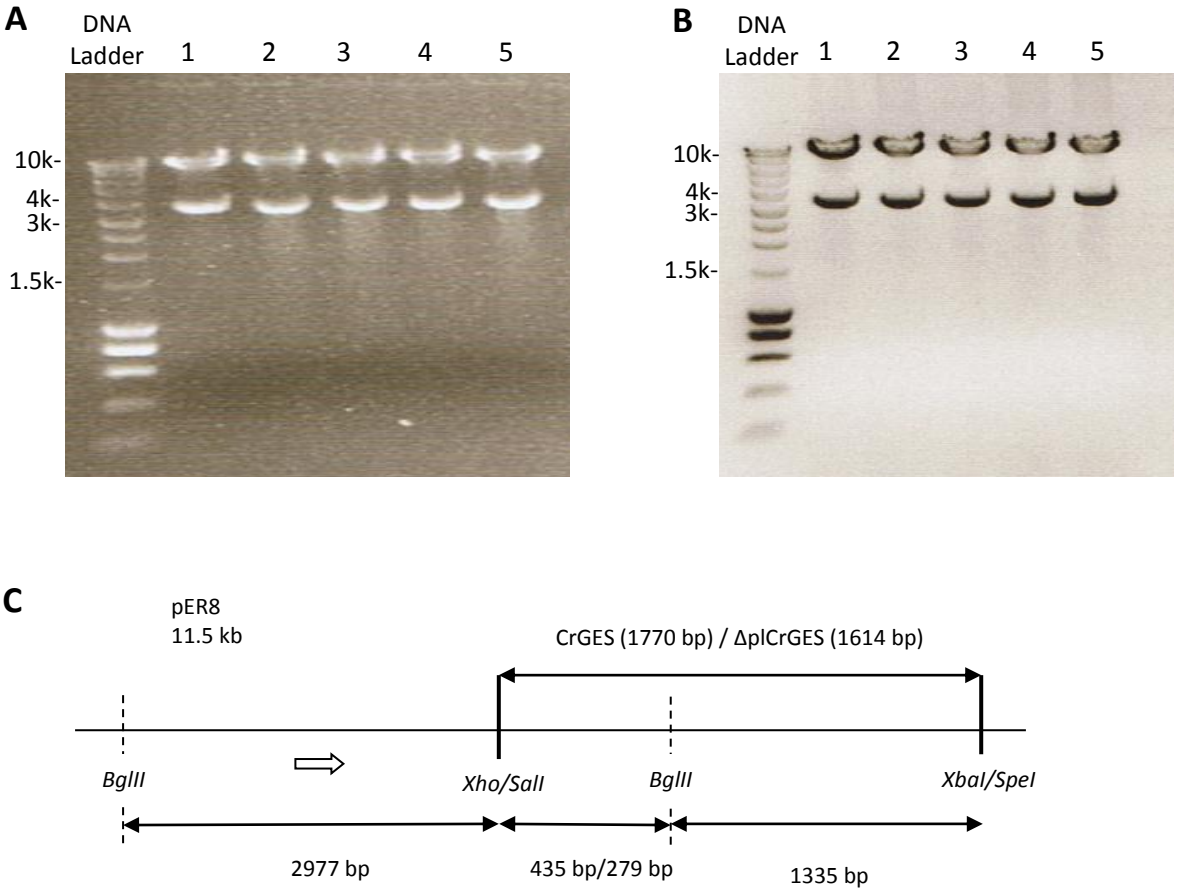


Supplement 5 Constructs of pRT101::CrGES (A) excised with *BglII/PstI* (1 – 5) and pRT101::ΔplCrGES (B) excised with *BglII/PstI* (1, 2) and *PstI* (3) give expected sizes of DNA bands as represented in the construct scheme (C).

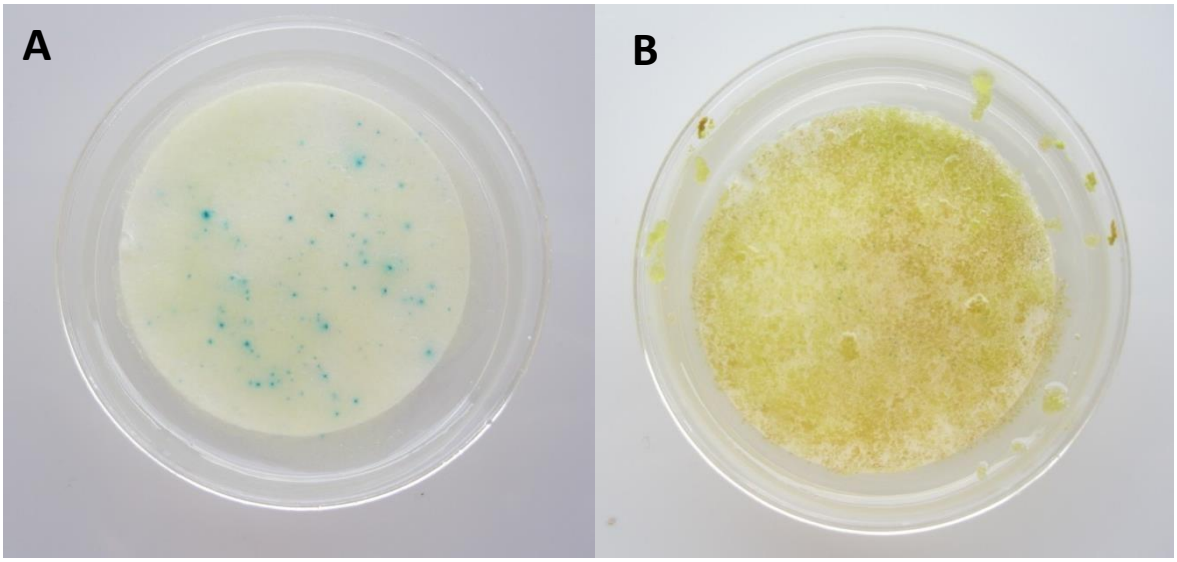




Supplement 6 Constructs of pER8::CrGES (A) and pER8::ΔplCrGES (B) excised with *BglII* give expected sizes of DNA bands as represented in the construct scheme (C).



Supplement 7 A study on transformation efficiency between cell-line MP183L (A) and CRPP (B) bombarded with 35S-GUS plasmid. Blue spots after histochemical staining with X-Gluc are caused by GUS activity.



Summary

Chapter 7

Catharanthus roseus is a valuable medicinal plant producing pharmaceutically important terpenoid indole alkaloids (TIA), such as the antihypertensive compounds ajmalicine and serpentine, and the anticancer dimeric/bisindole alkaloids vinblastine and vincristine. The plant produces very small amounts of those dimeric compounds and consequently the production is quite elaborate, which leads to high market prices. As commercial chemical synthesis of these compounds is not feasible due to their complex structures, particularly due to many chiral centers, an alternative production by biotechnological means using cell or organ cultures is of great interest. Cell cultures of *C. roseus* have been extensively studied for decades concerning different aspects of the formation of the TIA extending from genes to metabolite levels and including both biotic and abiotic factors. This makes *C. roseus* one of the best-studied medicinal plants and an important model system for plant cell biotechnology research. Nevertheless, TIA production by *C. roseus* cell cultures is still too low for commercialization unlike other plant cell products, producing e.g. shikonin (*Lithospermum erythrorhyzon),* paclitaxel (*Taxus brevifolia*), and ginsenosides (*Panax ginseng*). This is due to the low productivity of TIA in *C. roseus* cell cultures associated with several issues such as rate-limiting enzymes in the biosynthesis pathway, subcellular localization of metabolites and enzymes, and consequently the necessary inter- and intracellular transport, and competition for precursor for different metabolic pathways. All of these aspects require in-depth research to understand the conditions and regulation of the plant cells as a biofactory. Therefore, in this thesis, some aspects of TIA production were studied.

The study started with the characterizing nine *C. roseus* cell suspension lines which have been employed in various studies by our group (**Chapter 2**). In this study, the levels of TIA and their monoterpenoid precursors, sterols, and carotenoids were analyzed as they represent monoterpenoids (C10), triterpenoids (C30), and tetraterpenoids (C40), respectively, which potentially compete for the same carbon five precursors (C5). The results suggest that the geranylgeranyl diphosphate (GGPP; C20) pathway towards carotenoid production might compete with TIA biosynthesis as both pathways are derived from the same precursor, geranyl diphosphate (GPP; C10) coming from the MEP pathway. For channeling more

precursors to the TIA pathway, the branch point for C10 and C20 seems an interesting target for metabolic engineering. In addition, we searched for a candidate cell line with a high TIA-producing capacity for subsequent research. Among the *C. roseus* cell lines, the CRPP-type cell line turned out to be the best TIA-producing cell line.

There are various branches in the terpenoid pathway leading to the production of different terpenoid compounds, which suggests a competition for the C5 precursor pools. To study whether the stimulation of TIA production by certain signal molecules is due to a redistribution of precursors between the associated terpenoid pathways or to a total increase of precursor availability, the effects of jasmonic acid elicitation on different terpenoid pathways in *C. roseus* cells were analyzed. The production of the monoterpenoids (TIA; C10), triterpenoids (phytosterols; C30), and tetraterpenoids (carotenoids; C40), and distribution of C5 units into the terpenoid groups were evaluated. The results in **Chapter 3** show that TIA and carotenoid levels were increased upon JA elicitation, whereas the phytosterol levels remained constant. This indicates an enhanced availability of precursors through the MEP pathway. By evaluating the distribution of the MEP pathway derived C5 units, it seems that elicitation does not really change the ratio for the carotenoid and TIA pathways. In order to favor TIA production, it would be interesting to channel the increased availability of the C5 units from carotenoid to TIA. Furthermore, metabolomics profiling by ^{1}H-NMR showed metabolic alterations in JA-elicited cells, in which strictosidine, succinic acid, fumaric acid, and malic acid were increased, whereas sugar levels were decreased.

There is evidence of interaction between the IPP::DMAPP pools in the cytosolic mevalonate and the plastidial MEP pathways. The redirection of carbon resources indicate that IPP::DMAPP from the MEP pathway is ‘leaked’ to the cytosolic terpenoid pathway. In **Chapter 4**, a study was conducted to evaluate metabolic flows in different terpenoid pathways with specific attention to the distribution of C5-units into sterols (triterpenoids, C30), carotenoids (tetraterpenoids, C40), and TIA (monoterpenoids, C10). In addition, we wanted to investigate if there is indirect evidence that hypothetical leakage of MEP intermediates competes with precursor availability for the MEP derived products as the carotenoids and the TIA. By feeding the cytosolic mevalonate pathway with mevalonic acid, it is anticipated that the cytosolic routes are saturated and that leakage from the MEP pathway should be reduced, thus delivering more C5 units into carotenoids and TIA. Our results showed that feeding a low (0.2 mM) and a high (3.3 mM) concentration of mevalonic acid to the *C. roseus* cell suspension cultures increased the levels of sterols but did not increase the fluxes in the MEP pathway towards TIA and carotenoids.

Previous studies showed that geraniol might be a limiting upstream step of the monoterpenoid pathway. Therefore, various geraniol concentrations (0.32 – 1.62 mM) were fed to *C. roseus* cell suspension cultures (cell-line CRPP) to study the effect on the production of TIA and precursors (**Chapter 5**). We found that loganic acid and a large new peak as detected by HPLC-DAD were accumulated in a concentration and time-dependent manner. However, the production of strictosidine and down-stream TIAs seemed unaffected, whereas catharanthine and tabersonine levels decreased at the higher geraniol feeding concentrations (1.30 – 1.62 mM). A combination treatment of geraniol feeding (1.62 mM) and JA elicitation (102 μM) at the subsequent day significantly increased the level of strictosidine (68%) compared to only JA elicitation treatment at 72 hours after elicitation. However, the combination treatment did not further improve production of serpentine, catharanthine, and tabersonine. This shows that feeding geraniol to *C. roseus* cell suspension cultures leads to accumulation of loganic acid, whereas the combination with subsequent elicitation by jasmonic acid increases the flux towards strictosidine, but not to further down-stream TIA. In addition, signals of geraniol analogues were detected by NMR, which might be associated with the accumulation of new compounds in the geraniol fed cells.

Geraniol may be limiting as an upstream precursor in the monoterpenoid pathway due to limited production of the enzyme geraniol synthase or a limitation in the geraniol transport from plastid to cytosol. **Chapter 6** describes the overexpression of the *C. roseus'* geraniol synthase gene in the plastid (CrGES) and cytosol (ΔplCrGES) of *C. roseus* cells via biolistic transformation. We found that overexpressing CrGES and ΔplCrGES in non-TIA producing *C. roseus* cell cultures (cell-line MP183L) did not result in accumulation of TIA and iridoid precursors in the transformed cells. However, phenylalanine, tyrosine, valine, and leucine were found to be higher in the CrGES overexpressing lines, but lower in the ΔplCrGES overexpressing lines, compared to the control line transformed with the empty vector. Isoleucine and tryptophan were also higher in the CrGES than the ΔplCrGES overexpressing lines. Apparently, only primary metabolism is affected by CrGES and ΔplCrGES overexpression in the *C. roseus* cell suspension culture used in this study.

Perspectives

The commercial scale production of TIA production by plant cell cultures is hindered by the low yields. The plant cell factory consists of a complex network of biochemical processes, including trafficking, needed for the logistics of the biochemical pathways to function, e.g. to provide sufficient substrates, co-factors, and energy. Metabolic engineering

may be used to increase fluxes and overcome the bottle-necks in the TIA biosynthesis. However, this requires thorough understanding of the pathway regulation, including the biosynthetic genes and enzymes, regulation by transcription factors, subcellular localization of the biosynthetic enzymes, and intra- and intercellular transport of intermediates.

Studies have been focused on the elucidation of the upstream secoiridoid pathway which is considered as the rate limiting step for TIA biosynthesis. Our studies showed that there is also a limiting step to release strictosidine towards downstream TIA in *C. roseus* cell cultures and therefore further study is needed to elucidate the regulation of this step. Both precursor/substrate availability and gene expression indeed play critical roles in TIA production. Moreover, the biosynthesis pathway is spread over different cells as well as subcellular compartments and overexpressed enzymes should be targeted to the appropriate location.

Channeling more carbon into the TIA pathway and reducing the carbon flow to competitive pathway could improve TIA production but may interfere with other essential biochemical processes and may affect the plant cell growth. Instead of constitutive down regulation of competing steps, a new metabolic rerouting approach should be developed to realize temporal down regulation. In such an approach one should be able to control or switch on and off the regulation of the pathway of interest in the cell factory at a suitable time and maximize production of specific compounds, i.e. separate growth and production.

Transcription factors may act as master switches that control part or even full expression of a biosynthethic pathway. As overexpression of a single gene is often insufficient while overexpression of multiple genes is rather difficult, overexpressing a master activator could be a potential way to control overall gene expression in the pathway, thus increasing the biosynthetic flux and improving the yield of the desired compound(s). Although transcription factors that regulate the expression of certain genes in the TIA pathway have been cloned, none of them control the full pathway, so more studies are required to identify additional transcription factors regulating the TIA pathway.

Furthermore, the efficient machinery in the *Catharanthus* cells (e.g. our cell-line CRPP) to produce strictosidine could be exploited. Strictosidine is involved in the formation of many different types of alkaloids; by introducing heterologous alkaloid pathway genes into the *Catharanthus* cell-line the alkaloid production capacity could be highly extended. Alternatively, it can be isolated and employed as building block for chemical synthesis of alkaloid derivatives.

Reconstruction of a part or the whole TIA pathway in a heterologous host plant or other more productive systems like bacteria and yeast could be an alternative for the production of the alkaloids and to overcome the restrictions of production capacity in the plant cells, including the relative low generation time and biomass accumulation capacity. However, this requires the availability of the structural genes and knowledge on the regulation of the pathway and that the host can supply the main precursors. In addition, it remains to be assessed if the host supports the product pathway, and if the introduced pathway does not suffer from unexpected negative cross-regulation or competitive pathways in the (alternative) host. Most likely it means that not only structural genes of the TIA pathway need to be overexpressed or blocked, but also various regulatory genes as well as genes involved in competitive pathways, transport and storage of the products, in other words a complete resetting of the cell factory in a synthetic biology approach.

Samenvatting

Catharanthus roseus is een waardevolle medicinale plant die belangrijke farmaceutisch actieve terpeen indoolalkaloïden (TIA) produceert, zoals de bloeddrukverlagende verbindingen ajmalicine en serpentine, en de antikanker dimere/bisindool alkaloïden vinblastine en vincristine. De plant produceert zeer lage hoeveelheden van deze dimere verbindingen; hierdoor is de productie bewerkelijk wat leidt tot een hoge marktprijs. Aangezien chemische synthese als productiemethode voor deze verbindingen commercieel niet haalbaar is, vanwege hun complexe chemische structuur en vooral de vele chirale centra, is het van groot belang om een alternatief productieproces te ontwikkelen via een biotechnologische aanpak met plantencel- of orgaanculturen. Celculturen van *C. roseus* zijn al tientallen jaren intensief onderzocht aangaande verschillende aspecten betrokken bij de vorming van de TIA variërend van de genen, hun expressie en de niveaus van metabolieten geassocieerd aan de biosyntheseroute, inclusief het effect van biotische en abiotische factoren daarop. Hierdoor is *C. roseus* een van de best bestudeerde medicinale planten en een belangrijk modelsysteem binnen het plantencel biotechnologisch onderzoek. Desondanks is de TIA productie door *C. roseus* celculturen nog steeds te laag voor commercialisatie in tegenstelling tot andere met celculturen geproduceerde plantenverbindingen, zoals shikonine (*Lithospermum erythrorhyzon)*, paclitaxel (*Taxus brevifolia*), en ginsenosides (*Panax ginseng*). Dit is het gevolg van het lage biosyntheseniveau van TIA in *C. roseus* celculturen dat door verschillende aspecten beïnvloed wordt, zoals beperkte conversiecapaciteit van enzymen in de biosyntheseroute, de subcellulaire lokalisatie van metabolieten en enzymen met als gevolg daarvan een beperking door inter- en intracellulair transport, en competitie om precursor metabolieten tussen verschillende metabolische routes. Al deze aspecten vereisen diepgaand onderzoek om de specifieke condities en regulatie van processen in de plantencellen als productiefaciliteit te begrijpen. Een aantal van deze aspecten betrokken bij TIA productie zijn daarom tijdens dit promotie onderzoek bestudeerd en beschreven in dit proefschrift.

Het onderzoek begon met de karakterisatie van negen *C. roseus* celculturen die reeds voor verschillende studies binnen de onderzoeksgroep benut waren (**Hoofdstuk 2**). In deze studie werden de niveaus aan TIA inclusief hun monoterpeen precursors, sterolen en carotenoïden geanalyseerd, aangezien zij respectievelijk de metabole groepen van

monoterpenen (C10), triterpenen (C30) en tetraterpenen (C40) vertegenwoordigen, die potentieel in competitie zijn voor dezelfde isopreen (C5) precursor. De resultaten geven aan dat de metabole route naar geranylgeranyl difosfaat (GGPP; C20) en carotenoïde productie limiterend zou kunnen zijn voor TIA biosynthese, aangezien beide routes uitgaan van de precursor geranyl difosfaat (GPP; C10) die via de methylerythritol fosfaat (MEP) route gevormd wordt in de chloroplast. Het metabole vertakkingspunt tussen de C10 en C20 verbindingen lijkt daarom een interessant doel voor metabole en genetische modificatie. Daarnaast zochten we een kandidaat cellijn met een hoge TIA productie capaciteit voor vervolgonderzoek. Van de onderzochte *C. roseus* celculturen bleek de CRPP-type cellijn de beste, TIA-producerende cellijn te zijn.

Er zijn meerdere vertakkingen in de terpeen biosyntheseroute die naar verschillende terpeen groepen leiden en dit zou kunnen betekenen dat er competitie is om de voorraden aan isopreen C5 precursors. Om te bepalen of stimulatie van TIA biosynthese door signaal moleculen een gevolg is van herverdeling van precursors tussen geassocieerde terpeen routes danwel het gevolg van een totale toename aan precursor beschikbaarheid, is het effect van jasmonaat (JA) elicitatie op verschillende terpeen routes in *C. roseus* cellen geanalyseerd. De gehalten van de monoterpenen (TIA; C10), triterpenen (fytosterolen; C30) en tetraterpenen (carotenoïden; C40), en de verdeling van isopreen C5 eenheden zijn geëvalueerd. De resultaten in **Hoofdstuk 3** tonen aan dat de metabolietniveaus van de TIA en de carotenoïden toenamen na JA elicitatie, terwijl het gehalte aan fytosterolen constant bleef. Dit is een indicatie dat de precursors vanuit de MEP route in de chloroplast in verhoogde mate beschikbaar waren. Door de verdeling van isopreen C5 eenheden uit de MEP route te evalueren, bleek, dat de elicitatie de ratio van C5 eenheden tussen de caroteen en de TIA groepen niet lijkt te beïnvloeden. Het zou interessant zijn om de hogere beschikbaarheid aan C5 isopreen eenheden te kanaliseren van de carotenoïden naar de TIA om zo de TIA productie te bevoordelen. Naast de gerichte analyse van terpeen groepen, toonde ^{1}H-NMR analyse andere metabole veranderingen in JA geëliciteerde cellen aan, waaronder een toename aan strictosidine, barnsteenzuur, fumaarzuur, en appelzuur niveaus en een daling van de suiker niveaus.

Er zijn indicaties dat er een interactie is tussen de isopentenyl difosfaat (IPP) en dimethylallyl difosfaat (DMAPP) voorraden van de cytosolische mevalonaat en de plastidiale MEP routes. De herverdeling van de isopreen C5 precursors geeft aan, dat IPP::DMAPP uit de MEP route "uitlekt" naar de cytosolische terpeen route. In **hoofdstuk 4** is daarom de metabolietstroom tussen verschillende terpeen groepen geëvalueerd met specifieke aandacht

voor de verdeling van isopreen C5 eenheden naar sterolen en triterpenen (C30), carotenoiden (tetraterpenen; C30) en TIA (monoterpenen; C10). Verder is het hypothetische verlies van isopreen C5 eenheden uit de MEP route onderzocht als potentieel competitief element in de verdeling van MEP intermediairen ten opzichte van carotenoïden en TIA. Door middel van mevalonaat toevoeging aan celculturen om de cytosolische mevalonaat route te supplementeren, werd verwacht dat de cytosolische terpeen routes gesatureerd zouden worden, waardoor eventuele opname van isopreen eenheden uit de MEP route verminderd zou zijn, zodat verhoudingsgewijs meer isopreen C5 eenheden beschikbaar zouden blijven voor de carotenoïde en TIA biosynthese. De resultaten toonden aan dat het gehalte aan sterolen toenam bij toevoeging van mevalonaat aan de *C. roseus* celculturen tot een lage (0.2 mM) en een hoge (3.3 mM) eindconcentratie, maar dat er geen toename van metabolieten in de MEP route richting TIA en carotenoiden plaatsvond.

Eerdere studies hebben aangetoond dat de beschikbaarheid van geraniol een limiterende factor in de monoterpeen biosynthese zou kunnen zijn. Daarom is onderzocht wat het effect van geraniol op de biosynthese van TIA en monoterpeen precursors zou zijn door geraniol bij verschillende concentraties (0.32 – 1.62 mM) toe te voegen aan de *C. roseus* celculturen van de cellijn CRPP (**Hoofdstuk 5**). Als resultaat werd gevonden dat loganaat en een grote nieuwe piek, zoals waargenomen met HPLC-DAD, accumuleerden op een concentratie- en tijd-afhankelijke wijze. Echter, de productie van strictosidine en down-stream TIA leek onveranderd, terwijl catharanthine en tabersonine afnamen bij de hogere geraniol concentraties (1.30 – 1.62 mM). Een combinatie behandeling van geraniol supplementatie (1.62 mM) en JA elicitatie (102 μM) op de daaropvolgende dag, resulteerde 72 uur na elicitatie in een significant verhoogd strictosidine niveau (68%) ten opzichte van alleen JA elicitatie. Desalniettemin had de combinatie behandeling geen additioneel positief effect op productie van serpentine, catharanthine en tabersonine. Deze resultaten tonen aan dat geraniol supplementatie bij *C. roseus* celculturen leidt tot accumulatie van loganaat, terwijl additionele behandeling middels JA elicitatie leidt tot verdere conversie naar strictosidine, maar niet naar verdere down-stream TIA. Verder zijn met NMR analyse signalen van geraniol analogen gedetecteerd in de geraniol gesupplementeerde cellen, die mogelijk overeenkomen met de, door middel van HPLC-DAD, waargenomen nieuwe verbindingen.

De beperkte beschikbaarheid van geraniol als upstream precursor voor de monoterpeen route kan veroorzaakt worden door een gelimiteerde productie danwel activiteit van het enzym geraniol synthase en/of een beperking in het transport van geraniol van de

plastide naar het cytosol. **Hoofdstuk 6** beschrijft de overexpressie van het *C. roseus'* geraniol synthase gen in de plastiden (CRGES) en het cytosol (ΔplCrGES) van *C. roseus* cellen via biolistische transformatie. De overexpressie van CrGES and ΔplCrGES in de *C. roseus* cellijn MP183L, die onder standaard condities geen TIAs of precursors produceert, resulteerde niet in de accumulatie van TIAs of monoterpeen iridoid precursors in getransformeerde cellen. Echter, in vergelijking met de controle cellijnen, getransformeerd met een lege vector, bleken de gehalten aan phenylalanine, tyrosine, valine, en leucine hoger te zijn in de CrGES overexpressie lijnen, terwijl de waarden voor deze metabolieten in de ΔplCrGES overexpressie lijnen lager waren. Daarbij waren de niveaus aan isoleucine en tryptophan hoger in de CrGES dan in de ΔplCrGES overexpressie lijnen. Blijkbaar heeft de overexpressie van CrGES en ΔplCrGES alleen invloed op het primair metabolisme in de specifieke *C. roseus* cellijn gebruikt in deze studie.

Perspectieven

The TIA productie op commerciële schaal met cel-culturen wordt belemmerd door de lage opbrengsten. Het productieproces in de planten-cellen bestaat uit een complex netwerk aan biochemische processen, inclusief transport van betrokken elementen die nodig zijn om de logistiek van de biochemische processen op orde te houden, bijvoorbeeld met betrekking tot het aanvoeren van substraten, co-factoren en energie. Engineering van de metabole routes kan toegepast worden om de flux in de TIA biosynthese route te verhogen en om belemmeringen op te lossen. Dit vereist echter volledige kennis van de regulatie van de biosynthese route, inclusief kennis van de betrokken genen en enzymen, regulatie door transcriptiefactoren, subcellulaire localisatie van de enzymen en het intra- en intercellulaire transport van intermediairen.

Het onderzoek heeft zich gericht op de opheldering van het upstream secoiridoid deel van de TIA route, omdat de precursor beschikbaarheid in dit deel van de route als beperkende en snelheidsbepalende stap in de TIA biosynthese beschouwd wordt. De hier beschreven studies hebben aangetoond, dat er in de *C. roseus* celculturen ook een limiterende stap is in het vrijgeven van strictosidine naar de down-stream TIA en dat verder onderzoek nodig is om de regulatie van deze stap op te helderen. Zowel de precursor/substraat beschikbaarheid en expressie van betrokken genen spelen kritische rollen in de TIA productie. Daarbij is de biosynthese route verdeeld tussen verschillende celtypen in de plant en binnen de cellen ook nog in delen uitgesplitst over de verschillende subcellulaire compartimenten, waardoor het vereist is om tot over-expressie gebrachte enzymen correct te lokaliseren.

De TIA productie zou verbeterd kunnen worden als er meer isopreen C5 eenheden naar de TIA biosynthese route gekanaliseerd worden en als de flux naar competitieve routes verlaagd wordt, maar dit zou kunnen interfereren met andere essentiële biochemische processen die de groei en ontwikkeling van de plant beïnvloeden. In plaats van constitutieve repressie van competitieve routes, zou een nieuwe metabole aanpak ontwikkeld moeten worden waarmee temporele repressie gerealiseerd wordt. De beoogde aanpak zou het mogelijk moeten maken om de gewenste metabole routes en het productieproces in de cellen te controleren en de regulatie aan en uit te schakelen op het optimale moment en voor een bepaalde duur om zo specifiek de productie van de beoogde verbindingen te maximaliseren, en groei en productie te scheiden. Transcriptiefactoren spelen een rol als hoofdschakelaar die de expressie van genen in hele of delen van biosynthese routes controleren. Aangezien overexpressie van een enkel biosynthese-gerelateerd gen vaak onvoldoende is om effect te realiseren, terwijl de overexpressie van meerdere genen uit een route meestal lastig is, biedt de overexpressie van een activerende transcriptiefactor een alternatief waarmee algehele expressie binnen een biosynthese route gecontroleerd kan worden, zodat de biosynthese flux en opbrengst van gewenste metabolieten verhoogd kan worden. Een aantal transcriptiefactoren die betrokken zijn bij de regulatie van enkele TIA genen zijn gecloneerd, maar geen van hen controleert alle genen uit de biosynthese route. Derhalve is meer onderzoek nodig om additionele transcriptiefactoren te identificeren die de TIA biosynthese route reguleren.

Verder kan de efficiëntie van de *Catharanthus* cellen (bijvoorbeeld van de CRPP cellijn) om strictosidine te produceren, benut worden. Strictosidine is in verschillende plantensoorten betrokken bij de vorming van veel verschillende alkaloïden; door introductie van heterologe genen naar de *Catharanthus* cellen kan de productiecapaciteit voor alkaloïden enorm uitgebreid worden. Als alternatief kan de strictosidine geïsoleerd worden om als component te dienen voor verdere chemische synthese van alkaloïd-derivaten.

Reconstructie van een deel of de hele TIA biosynthese route in een heterologe, plantaardige gastheer of een ander hoogproductief bacterieel of gist model zou een alternatief bieden voor de productie van alkaloïden om de restricties in de productiecapaciteit van plantencellen op te lossen, inclusief de beperkingen met betrekking tot lange generatietijd en lage biomassa productie van plantencellen. Deze alternatieve aanpak vereist de beschikbaarheid van alle structurele biosynthese genen, kennis van de regulatie van de biosynthese route en het vermogen van de gastheer om alle precursors te produceren. Daarbij moet geëvalueerd worden of de gastheer productvorming kan faciliteren en of de

geïntroduceerde route niet onverwacht gehinderd wordt door endogene regulatoire factoren en competitieve routes. Het meest plausibel is, dat in dit scenario niet alleen structurele genen van de TIA route tot overexpressie gebracht of geblokkeerd moeten worden, maar dat ook meerdere regulatoire genen, genen van competitieve routes, of betrokken bij transport en opslag van de producten aangepast moeten worden; kortom het hele productieproces in de cellen moet ge-reset worden in een synthetisch biologische aanpak.

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Curriculum Vitae

Mohd Zuwairi bin Saiman was born on the 9th of February 1983 in Malacca, Malaysia. After finishing secondary education in Sekolah Menengah Sains Muzaffar Syah (Muzaffar Syah Science Secondary School), he attended a science matriculation program in University of Malaya (UM), Kuala Lumpur. In 2002, he continued his bachelor degree in the same university and he obtained a BSc (Honors) in Biotechnology in 2006. His undergraduate research project was about physiological studies of micropropagated banana plants (*Musa acuminata* var. berangan) from *in vitro* to *ex vitro* condition. After graduation, he worked as a research assistant in Plant Biotechnology Incubator Unit in UM. He conducted a research dealing with mass propagation and production of bioactive compounds of *Zingiber zerumbet* (pinecone ginger) cell cultures in bioreactors. In 2007, he was awarded a scholarship and an academic staff training scheme from Ministry of Education Malaysia and UM, respectively, to pursue his postgraduate studies. He continued his MSc in the Pharmacognosy/Plant Biotechnology Department, Leiden University with research on the secondary metabolites production in cell and root cultures of the medicinal plant *Catharanthus roseus* and *Gynura procumbens*. He finished the MSc degree in July 2009 and five months later, he started his PhD in Natural Products Laboratory, Institute of Biology, Leiden University. After getting PhD, he will be serving UM as a lecturer and a researcher.

Publications

Mohd Zuwairi Saiman, Natali Rianika Mustafa, Anna Elisabeth Schulte, Robert Verpoorte, Young Hae Choi (2012) Induction, characterization, and NMR-based metabolic profiling of adventitious root cultures from leaf explants of *Gynura procumbens*. Plant Cell, Tissue and Organ Culture 109: 465–475

Mohd Zuwairi Saiman, Natali Rianika Mustafa, Barbora Pomahačová, Marianne Verberne, Robert Verpoorte, Young Hae Choi, Anna Elisabeth Schulte (2014) Analysis of metabolites in the terpenoid pathway of *Catharanthus roseus* cell suspensions. Plant Cell, Tissue and Organ Culture 117: 225–239