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.