Summary

Plants produce a wide variety of secondary metabolites, which play a role in the interaction of plants with their environment. For example, they may act as attractants for pollinators, or as defense compounds against pathogens, insects, herbivores or abiotic stresses (e.g. UV-light, high-salinity and drought), or as signal compounds. Salicylic acid (SA) is a signal compound for so-called systemic acquired resistance (SAR). It belongs to C6C1 group of compounds, which structurally consists of a carboxyl group attached to an aromatic ring. SA derives from the shikimate pathway, a very important pathway in plants linking the carbohydrate metabolism to aromatic compounds metabolism. It consists of seven steps and it is the start for the biosynthesis of a broad spectrum of secondary metabolites e.g. phenolic compounds and alkaloids. The end product of the shikimate pathway is chorismate, which represents an important branching point, as it is a substrate of five different enzymes (reviewed in Chapter 2). The products of these branches are among others, phenylalanine, anthranilate, tryptophan, p-aminobenzoic acid, p-hydroxybenzoic acid, 2,3-dihydroxybenzoic acid as well as SA. From the products a broad variety of secondary metabolites is derived. Unraveling the pathways leading to the C6C1 compounds in plants by isolation and characterization of the responsible enzymes and cDNAs are important not only for a basic understanding of SAR but also for application in metabolic engineering.

Phenolic compounds such as simple phenolics (C6), C6C1 compounds, simple phenylpropanoids (C6C3 compounds) and flavonoids (C6C3C6 compounds) are very common in plants and often increase in level upon a pathogenic attack. Also in Catharanthus roseus plants and cell cultures the presence of phenolic compounds was reported in several studies (reviewed in Chapter 3). Catharanthus roseus is a source of terpenoid indole alkaloids (TIAs) e.g. vincristine and vinblastine, the potent antitumor agents. In order to gain more insight in the flux through the pathway leading to desired metabolites like TIAs, studies about the biosynthetic pathways of other secondary metabolites and their regulation are necessary. The regulation of metabolic networks employs signal compounds such as SA, jasmonate (JA) and ethylene (ET). Different pathways can be activated depending on the plant environment or kind of biotic- or abiotic stress that a plant experiences. Differences in
the pathways activated, are not only found between species but also between plants, plant organs, cell cultures or seedlings of a species. For example elicitation of *C. roseus* cell cultures by *Pythium* extracts results in an increase of SA, 2,3-DHBA and tryptamine.

Since increased levels of SA and 2,3-DHBA (Budi Muljono, 2001) paralleled an increase in the isochorismate synthase (Budi Muljono et al., 2002) in *C. roseus* suspension cell culture upon elicitation by *Pythium* extract, the presence of the microbial pathway leading to SA in this species is plausible, as it was proposed by Verberne et al. (2000). Microorganisms convert chorismate to SA by isochorismate synthase (ICS) and isochorismate pyruvate-lyase (IPL), whereas SA in plants is thought to derive from phenylalanine, which involves many steps. Budi Muljono et al. (2002) proved the involvement of the microbial pathway for 2,3-DHBA by means of a retrobiosynthetic study. To proof this pathway also for SA, a high producing cell line is required because usually SA is produced in trace amounts only. Using a quantitative analysis HPLC method for SA developed by Verberne et al. (2002), we investigated the levels of SA upon elicitation with *Pythium* extract in some *C. roseus* cell lines for finding a high-SA-producing cell line necessary for labeling experiments (Chapter 4). We found that *C. roseus* grown in Murashige & Skoog medium without growth hormones (a wild-type A12A2 line) produced the highest level of total SA (free SA and SA-glucoside).

Besides the elicitation with *Pythium* for activation of the SA-pathway and for increasing the SA level, purification of SA is still necessary since the increased-level of SA still yields minor quantities as compared to other compounds, and consequently the signals of these compounds will interfere with SA-signals in the NMR spectra. We developed an anion exchange method to purify SA, consisting of a Dowex 1WX2 (100 mesh) as the resin, 0.25 mM sodium-phosphate pH (7.0-7.5) as the washing solvent and 0.3 M HCl in 60% of AcCN as the counter ion solution. It was observed that AcCN is better than MeOH as the organic solvent used in the counter ion solution. This system provides a good result as a single step purification of SA from plant cell cultures as observed in the (400 MHz) $^1$H-NMR spectra (Chapter 5).

The availability of an efficient purification method for SA opened the possibility to perform the labeling studies. [1-$^{13}$C]-D-Glucose was used for the labeling experiment (Chapter 6). This labeled precursor is commonly used for retrobiosynthetic studies e.g. in yeasts and plants (Werner et al., 1997) including in *C.
*rosegus* cell cultures (Contin *et al.*, 1998; Budi Muljono *et al.*, 2002). Purification of a labeled crude extract of *C. rosegus* elicited cells using the ion exchange chromatography system followed by a Sephadex-LH 20 column resulted in a well-purified enriched-SA extract as detected in the HMBC and $^{13}$C-NMR spectra. The inverse gated $^{13}$C-NMR method using a 600 MHz NMR spectrometer with a relatively high number of scans (36864) was used for the quantitative $^{13}$C-NMR analysis of labeled-SA. The results showed a clear asymmetry of incorporation at C-2 and C-6, and a relatively low incorporation at C-7 of SA as well as of 2,3-DHBA, pointing to the isochorismate pathway as the responsible pathway for both SA and 2,3-DHBA in the *C. rosegus* cell culture elicited by *Pythium* extract. The different patterns of label between SA and 2,3-DHBA shows that both compounds were synthesized at a different time and/or localization. Further investigations to phenylalanine derived compounds such as the phenylpropanoids found in our elicited cells, and an analysis of the metabolome of the cells should provide more insight in the pathways leading to other compounds derived from phenylalanine in *C. rosegus*.

Metabolomics is a tool that should be able to give more information about possible metabolic changes after elicitation. By using NMR-based metabolomics, it is possible to get a general holistic view on all major compounds including both primary and secondary metabolites from a large variety of biosynthetic pathways (Kim *et al.*, 2006). We applied this approach for following the effect of SA on *C. rosegus* cells over a 72 h period (*Chapter 7*). We found that treatment of the *C. rosegus* cells with 0.5 M sodium-SA changed the metabolites profile through time compared to non-treated cells. In the SA-treated cells, the highest levels of sugars were found at 0 h and these levels subsequently decreased to zero after 72 h. The changes in the levels of some aliphatic amino acids and organic acids were also observed. SA signals disappeared at 48 h but at the same time the signals of 2,5-dihydroxybenzoate glucoside (2,5-DHBAG) increased significantly. This might be a catabolic product of SA as previously reported by Shimoda *et al.* (2002). However, we could not find the signals of TIAs and also the TIA precursors loganic acid and secologanin due to the very low levels of the compounds or total absence. Detection of compounds present in low levels needs further investigation using other analytical methods for example HPLC-DAD and/or LC-MS/GC-MS. Metabolic profiling of *C. rosegus* suspension cells elicited by jasmonate, *Pythium* extract or other elicitors would be necessary for further investigation about activation of different pathways by different elicitors.
It has been shown in this thesis that metabolic profiling by $^1$H-NMR in combination with Principal Component Analysis can provide information about the metabolic changes in the cells in a time course after elicitation. However, for mapping a pathway the involvement of certain intermediates has to be confirmed by $^{13}$C-NMR analysis of the labeled compounds after feeding labeled precursors.

**Perspectives:**

An efficient (routine) rational metabolic engineering in plants is impossible without knowing the structure of the plant metabolic network involved including the fluxes through the pathways (Ratcliffe and Shachar-Hill, 2005). The recent successful metabolic engineering of microorganisms is due to the more simple structure of their metabolic networks. However, most plant secondary metabolites cannot be produced using microorganisms. To be able to understand the structure of plant metabolic networks in all its complexity would be a breakthrough since complexity is found at all levels of biological organization. The demand to learn to understand the structure of metabolic networks is huge for not only to fulfill the human curiosity, but also for the potential important applications of such knowledge. The blueprint of the phenotype lies in the genes, however, the final phenotype is the result of the interaction of the organism with its environment, genomics and transcriptomics alone can not only explain the phenotype. These omics plus proteomics and metabolomics are necessary to achieve this goal though many studies showed that there is no direct parallel correlation between transcription, enzyme activity and the corresponding metabolites. Metabolic profiling and metabolic flux analysis (fluxomics) are important tools for unraveling the pathways part by part, including assessment of the performance of parts of the network. Tracer experiments using a stable isotope to measure fluxes through pathways have been quite extensively performed for measuring fluxes in primary metabolism. Even though the difficulties one has to face, for example the low levels of secondary metabolites, it is now the time to start to apply fluxomics on both primary and secondary metabolites in plants to realize the final goal; mapping the total plant metabolic network, including its regulation.