Retrobiosynthetic study of salicylic acid in Catharanthus roseus cell suspension cultures

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Chapter 1

General Introduction

A plant is a living organism able to convert inorganic material into organic molecules necessary for the life of plant itself and serves as food for e.g. insects, animals and humans. Plants also provide medicines, food additives, flavors, fragrances, pigments, insecticides, paper, fibers, rubber and many other commodities. However, our knowledge about plants with their enormous diversity is still limited in many aspects. So, still many novel products might be obtained from plants, however, this is hampered by the rapid loss of plant diversity on earth due to e.g. deforestation. The sustainable exploitation of plants for food and medicines requires extensive knowledge about plants. As the world's population grows to an estimated 9 billions people in 2050 (Cordell, 2002), the availability of food and medicines for all people in the future should be a concern to all of us. Up to now, plants remain a primary source of medicines for most people in the world (Cordell, 2002). Therefore, research in plant science is of great importance for human health, both for the production of healthier food and for development and production of medicines.

The secondary metabolites that are the source for e.g. pharmaceuticals, food additives or flavors, are species-specific and play a role in the interaction of a plant with its environment (Verpoorte, 1998). Examples are compounds aimed at attraction of pollinators (e.g. insects) or to defend against invaders (e.g. pathogens). An example of the role of secondary metabolism, which is also the basis of the present study, is the plant defense against infections with viruses or microorganisms. The production of secondary metabolites for plant defense such as phytoalexins can be a result of so-called systemic acquired resistance (SAR), an inducible broad-resistance to pathogens. SAR is activated after the formation of a necrotic lesion in leaves as part of the hypersensitive response (HR) to an infection. SAR is associated with the

expression of SAR genes responsible for SAR proteins; many belong to the class of pathogenesis-related (PR) proteins e.g. acidic PR1 proteins, which have antimicrobial activity (reviewed by Ryals *et al*., 1996). Besides the expression of SAR genes as a marker of SAR, SAR is also known to employ salicylic acid (SA) as a signal compound. Another inducible systemic resistance employing other signal compounds like e.g. jasmonate (JA) and ethylene (ET) is called induced-systemic resistance (ISR), which is known to activate genes encoding e.g. proteinase inhibitors and defensins (van Wees *et al*., 2000). Nitric oxide (NO), a signal compound for immune responses in animals, was shown also to mediate plant defense responses against pathogens (Durner and Klessig, 1999). Interaction between signal compounds can occur in a plant generating a systemic resistance as it was reviewed by e.g. Pieterse *et al*. (2001) and Kunkel and Brooks (2002). For example, SA and JA can activate the same genes in *Arabidopsis*. Several genomic studies showed that both SA signaling- and JA signaling pathways need activation of the *NPR1* gene (also called *NIM1* or *SAI1*), which was originally discovered as a key regulatory gene for activation of *PR-1* gene expression that functions downstream of SA in the SAR pathway (reviewed by van Wees *et al*., 2000). In *Arabidopsis*, the cytoplasmic-located NPR1 and WRKY70 (a component downstream of NPR1) mediates the cross-talk between the SA and the JA-signaling pathways. WRKY70 is activated by SA but repressed by JA, possibly functioning as a signal integrator from the mutually antagonistic SA and JA pathways (reviewed by Garcion and Métraux, 2006). Extensive genomic studies about SAR in some plant species and particularly in *Arabidopsis thaliana*, showed that the SAR pathway is a complex network (Shah, 2003; Garcion and Métraux, 2006). Thus, in generating systemic resistance, plants may employ multiple signals of different compounds such as SA, JA, ET or NO. Studies with *Catharanthus roseus* plants or cell cultures showed that biotic- or abiotic stress could lead to the production of different secondary metabolites as a defense response, which might employ different signal compounds (reviewed in Chapter 3).

In the plant defense, SA thus plays a key role. However, the biosynthesis of SA is still a matter of debate; several biosynthetic pathways exist in nature (see Chapter 6). For many years it was thought that in plants SA was derived from phenylalanine via benzoic acid. However, Verberne *et al*. (2000) showed that it is possible to introduce the microbial SA biosynthesis via the isochorismate pathway in plants. Wildermuth *et al*. (2001) showed the involvement of the isochorismate synthase (ICS) gene in

Arabidopsis in the biosynthesis of SA, but so far no direct chemical evidence exists for this pathway in plants. Budi Muljono *et al*. (2002) showed that in *C. roseus* the closely related dihydroxybenzoic acid is derived from isochorismate by a retrobiosynthetic study. As this plant cell culture also produces small amounts of SA after elicitation, this cell culture seemed an excellent model for studying the SA biosynthesis.

Labeling experiments with a stable ^{13}C isotope are commonly used to map metabolic pathways since ^{13}C is not radio-active and nuclear magnetic resonance spectrometry (NMR) analysis allows determining the precise site of the label in a molecule. Natural abundance of ^{13}C is 1.1%, so on a labeling of 1.1% will lead to a doubling of the percentage of the carbon being labeled and consequently to a clear increase of the signal concerned. In this way relative labeling of all carbons in a molecule can be measured. A high-level of incorporation of the label is important for a successful of labeling experiment. This is determined by several factors such as plant species, kind of labeled-precursor administered (the number of potential metabolic steps for converting the administered precursor to the target compound), the level of the precursor in the medium, kind- and amount of the cells, the activation of the target pathway (due to biotic- or abiotic stresses), the metabolic stability of the target compound, etcetera. Early precursors such as [1-^{13}C]-D-glucose or [U-^{13}C]-D-glucose are often used for the study of a biosynthetic pathway in yeast or plants (e.g. Werner *et al*., 1997). *Catharanthus roseus* suspension cultures have been shown to be a suitable model for retrobiosynthetic studies of iridoids (Contin *et al*., 1998) and 2,3-DHBA (Budi Muljono *et al*., 2002).

Aim of the thesis

The aim of this study is to map the biosynthetic pathway of salicylic acid in *C. roseus* cell suspension culture elicited by *Pythium aphanidermatum* extract using a retrobiosynthetic approach.

Outline of the thesis

SA is a C6C1 compound derived from chorismate either via the precursor phenylalanine or isochorismate. A review about chorismate-derived C6C1 compounds with the emphasis on the biosynthetic pathways is presented in **Chapter 2**. SA belongs to the phenolic compounds, a group of secondary metabolites that is widely

distributed in plants and often their production is increased under stress conditions. The phenolic compounds present in *C. roseus*, their biosynthetic pathways and regulation are discussed in **Chapter 3**. The level of SA in some lines of *C. roseus* cell suspension cultures after elicitation with *Pythium* extract were studied (**Chapter 4)** in order to select a high-SA producing cell line that would be used as a model in the labeling experiment. **Chapter 5** deals with development of a purification method of SA to allow analysis of the trace amonts in the cells by NMR. The results of the labeling experiments using the early-precursor [1-^{13}C]-D-glucose are reported in **Chapter 6**. SA is an important signaling compound in SAR, the effect of exogenous SA on the metabolites in a *C. roseus* suspension cells during a time course is reported in **Chapter 7**. Finally, a summary is presented at the end of the thesis.