Retrobiosynthetic study of salicylic acid in Catharanthus roseus cell suspension cultures

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Chapter 2

Chorismate-derived C6C1 compounds in plants

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2.1 Introduction

The secondary metabolites are the products of interaction of the producing organism with its environment and have a restricted occurrence. Many have economical importance as, e.g. drugs, antioxidants, flavors, fragrances, dyes, insecticides and pheromones (Verpoorte *et al*., 2002). Secondary metabolites can be classified according to their biosynthetic building blocks or their carbon skeleton. The C6C1 compounds are compounds having an aromatic six-carbon ring with one carbon attached. They are generally derived from the shikimate pathway (Dewick, 2002).

The shikimate pathway, restricted to microorganisms and plants, includes seven metabolic steps, starting with phosphoenolpyruvate and D-erythrose-4-phosphate, and ending with chorismate (an important metabolic branch-point) (Figure 2.1). All enzymes involved have been purified and the cDNAs characterized from some prokaryotes and eukaryotes (Herrmann and Weaver, 1999). In plants, the pathway is localized in plastids.

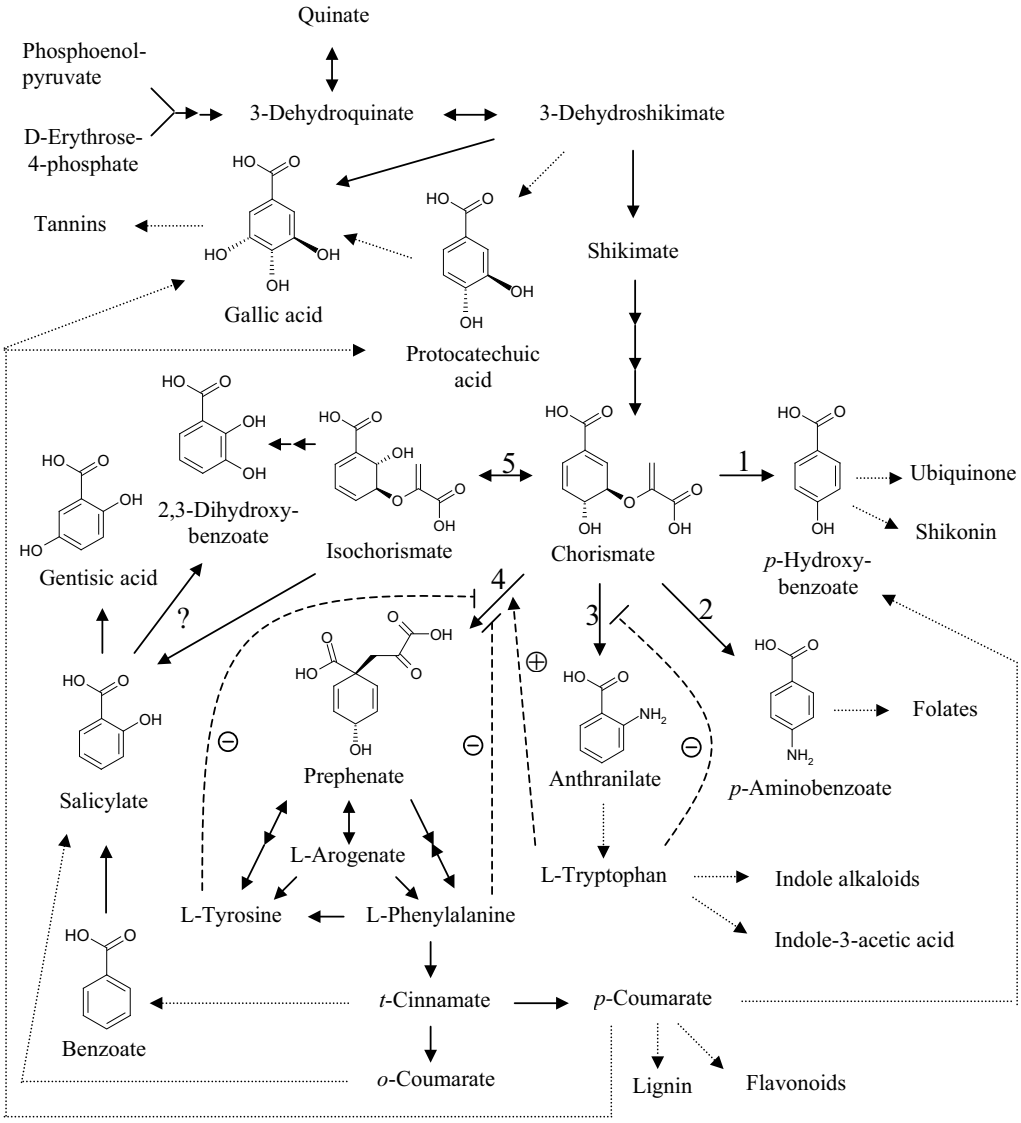

Figure 2.1 The biosynthetic pathway of chorismate/isochorismate derived-C6C1 compounds. 1 = chorismate pyruvate-lyase; 2 = *p*-aminobenzoate synthase; 3 = anthranilate synthase; 4 = chorismate mutase; 5 = isochorismate synthase. The dashed lines with + and – indicate feedback activation and inhibition respectively. A dotted line means multi-step reactions.

Gallic acid and protocatechuic acid (3,4-dihydroxybenzoic acid) are C6C1 compounds that can derive from either shikimate pathway (by dehydration and dehydrogenation of 3-dehydroshikimic acid) or phenylalanine pathway (via 3,4,5-trihydroxycinnamic acid) (Torssell, 1997; Ossipov *et al*. 2003). Gallic acid can also derive from orsellinic acid via the polyketide pathway by decarboxylation and oxidation (Torssell, 1997). This review will focus on chorismate-derived C6C1 compounds in plants, including anthranilate, *p*-aminobenzoate, *p*-hydroxybenzoate, salicylate and 2,3-dihydroxybenzoate.

2.2 Anthranilate

Anthranilate is the product of anthranilate synthase (AS, EC 4.1.3.27), the first enzyme of the tryptophan biosynthesis. The flux through this pathway is controlled by feedback inhibition by tryptophan on AS (Li and Last, 1996). AS is a key regulator for alkaloid accumulation induced by elicitors in *Ruta graveolens* (Bohlmann *et al*., 1995) and it may be a rate-limiting enzyme in the biosynthesis of avenanthramides, indole phytoalexins in oats (Matsukawa *et al*., 2002).

AS holoenzymes are characterized as tetramers consisting of two α- and two β-subunits, encoded by separate nuclear genes, synthesized in the cytosol and transported into the plastid to obtain the mature active form (Zhang *et al*., 2001). Two genes encoding ASα subunits (*ASA1* and *ASA2*) were isolated from *Arabidopsis thaliana* and found to be functional by complementation in yeast and *E. coli* (Niyogi and Fink, 1992). The overexpression of the *Ruta graveolens* ASα isozymes in *E. coli* revealed the presence of a tryptophan feedback-insensitive ASα1 and a sensitive ASα2 enzyme (Bohlmann *et al*., 1996). Transformation of a 5-methyl tryptophan-resistant tobacco gene (*ASA2*) into *Astragalus sinicus* (a forage legume) resulted in an increased level of tryptophan (Cho *et al*., 2000). An *Arabidopsis* feedback-resistant ASα gene (a mutated *ASA1*) was transformed into *Catharanthus roseus* providing hairy roots with increased levels of tryptophan, tryptamine and the indole alkaloid lochnericine (Hughes *et al*., 2004). Relocating a native tryptophan feedback-insensitive gene from the nucleus to the plastid genome resulted in transplastomic tobacco plants with greatly increased tryptophan levels but normal phenotype and fertility, showing the advantage of plastid transformation compared to nuclear transformation (Zhang *et al*., 2001). Replacing aspartate with asparagine at a certain

position in *A. thaliana* (Li and Last, 1996) or *Oryza sativa* ASα (Tozawa *et al*., 2001), resulted in lower sensitivity for tryptophan inhibition. Sensitivity for tryptophan inhibition can also be due to a mutation in a regulator gene of the AS gene's expression (Ishikawa *et al*., 2003). The genes encoding the rice plastidial ASβ subunits have been characterized (Kanno *et al*., 2004). Both ASβ subunits are assembled with the mature forms of the ASα subunits.

2.3 Salicylic acid

Salicylic acid (SA) has several roles in plants (Raskin, 1992) including the induction of systemic acquired resistance (SAR) as response to pathogens. SA-dependent SAR is characterized by the increase of SA and its conjugates and pathogenesis related (PR) proteins (Ryals *et al*., 1996).

SA in plants is thought to be derived from the phenylalanine pathway by cinnamic acid chain shortening, either through a β-oxidative or a non-oxidative pathway. Some steps have been identified, others not yet (Verberne *et al*., 1999). The enzyme (benzoic acid 2-hydroxylase) converting benzoic acid (BA) into SA has been identified (Leon *et al*., 1995). The non-oxidative pathway to BA does not function in cucumber (*Cucumis sativus*) and *Nicotiana attenuata* (Jarvis *et al*., 2000). In microorganisms, SA biosynthesis involves isochorismate synthase (ICS, EC 5.4.99.6), converting chorismate into isochorismate, and isochorismate pyruvate lyase (IPL) providing SA (reviewed by Verberne *et al*., 1999). Verberne *et al*. (2000) suggested that plants may utilize this pathway and they introduced the microbial-isochorismate SA pathway into tobacco resulting in increased-SA levels and enhanced resistance to tobacco mosaic virus. Wildermuth *et al*. (2001) found evidence for a SA isochorismate pathway. The *Arabidopsis sid2* mutant unable to produce chloroplast-localized ICS1 exhibited a remarkable lower level of SA after infection and a reduced resistance against pathogens. Chong *et al*. (2001) showed that the SA accumulation in elicited tobacco cells required de novo BA synthesis from *trans*-cinnamic acid, though, instead of BA, the benzoyl-glucose was the likely intermediate. The pathway from *trans*-cinnamic acid to SA via BA is involved in the stress-induced flowering of *Pharbitis nil* (Hatayama and Takeno, 2003).

The catabolism of SA is mainly through glucosylation by SAglucosyl transferase, which occurs presumably in the cytoplasm and subsequently accumulated in the

vacuoles. The uptake of SAG into vacuoles may involve different mechanisms in different plant species. For example, in soybean (*Glycine max*), the ATP-binding cassette (ABC) transporter is involved, whereas in the red beet it is the H^+-antiport mechanism (Dean and Mills, 2004). In a *Catharanthus roseus* cell suspension culture, SA was catabolized by a hydroxylation into 2,5-dihydroxybenzoic acid (gentisic acid) followed by a glucosylation of the newly introduced phenolic hydroxyl group. The 55 kDa hydroxylase and the 41 kDa regiospecific glucosyltransferase have been isolated by Shimoda *et al*. (2004) and Yamane *et al*. (2002).

2.4 2,3-Dihydroxybenzoate

2,3-Dihydroxybenzoate (2,3-DHBA) is in microorganisms derived from isochorismate (Young *et al*., 1968). SA and 2,3-DHBA are precursors of siderophores such as enterobactin and pyocheline. This pathway involves ICS, 2,3-dihydro-2,3-DHBA synthase and 2,3-dihydro-2,3-DHBA dehydrogenase. 2,3-DHBA may derive from SA by hydroxylation (reviewed by Budi Muljono, 2002). 2,3-DHBA is produced in *Catharanthus roseus* cell cultures after elicitation with fungal cell-wall preparations and parallels an increase in activity of ICS (Moreno *et al*. 1994). The ICS protein and its cDNA were obtained from *C. roseus* cell cultures (van Tegelen *et al*., 1999). This ICS has 57% homology with the ICS1 of *A. thaliana* and 20% homology with bacterial ICS (Wildermuth *et al*., 2001). A retrobiosynthetic study with *C. roseus* suspension cells fed with [1-^{13}C]glucose confirmed the intermediacy of isochorismate in 2,3-DHBA biosynthesis (Budi Muljono *et al*., 2002).

2.5 *p*-Hydroxybenzoate

p-Hydroxybenzoate (4HB), a precursor of shikonin, is formed via the phenylpropanoid pathway (Löscher and Heide, 1994). It is also a precursor of ubiquinones formed directly from chorismate by chorismate pyruvate-lyase (CPL) in bacteria or from both pathways in eukaryotic microorganisms (Meganathan, 2001). The *ubiC* gene encoding CPL of *E. coli* was overexpressed in tobacco resulting in high CPL activity and increased level of 4HB as β-glucosides (4HBG, 0.52% DW) derived from the introduced pathway (Siebert *et al*., 1996). Using the same constructs, only 20% of the total 4 HBG produced in *Lithospermum erythrorhizon* employed this pathway (Sommer *et al*., 1999). Transformation using a strong $(ocs)_3$mas promoter

did not change the level of 4HBG compared to the control cultures, but 73% of total 4HBG was derived from the introduced pathway (Köhle *et al*., 2002). Whilst, introducing this construct into tobacco and potato led to 5.1% (DW) of 4HBG in tobacco cell cultures and 4.0% DW in the leaves of potato shoots. These amounts correlated with CPL activity and are the highest for artificial secondary metabolites ever reached by genetic engineering in plants. It did not affect growth, proving the large capacity of the plastidial shikimate pathway (Köhle *et al*., 2003). *UbiC* without a transit peptide provided much lower levels of 4HB derivatives (Sommer and Heide, 1998). In *L. erythrorhizon*, 4HBG was accumulated in vacuoles. The vacuolar transport of 4HB and of *p*-hydroxycinnamic acid in red beet requires glucosylation and employs an H^+-antiport mechanism, the same transport used by 5-hydroxychlorsulphuron (a herbicide)-glucoside (Bartholomew *et al*., 2002).

2.6 *p*-Aminobenzoate

p-Aminobenzoate (PABA) is the precursor of folic acids (folates). Folates are cofactors in "one carbon" transfer reactions as e.g. in the biosynthesis of some nucleotide bases (Scott *et al*., 2000). The conversion of chorismate into PABA in microorganisms is catalyzed by *p*-aminobenzoic acid synthase, EC 4.1.3.-. This enzyme consists of three subunits. The large subunit (aminodeoxychorismate synthase) encoded by *pabB*, converts chorismate into aminodeoxychorismate (ADC), the small subunit encoded by *pabA* is a glutamine amidotransferase and the third subunit (aminodeoxychorismate lyase) encoded by *pabC*, converts ADC into PABA and pyruvate (Viswanathan *et al*., 1995).

Sulfonamides are PABA analogues inhibiting dihydropteroate synthase (DHPS), the enzyme converting PABA into 7,8-dihydropteroate (Scott *et al*., 2000). DHPS is the key regulator of the folate biosynthetic pathway (Mouillon *et al*., 2002). The cDNA was recently purified and characterized from pea leaves. The presence of a putative mitochondrial transit peptide of 28 amino acids in the single copy gene, indicates the mitochondria as the site of 7,8-dihydropteroate synthesis (Rebeille *et al*., 1997), thus requiring transport of PABA across the plastidial- and mitochondrial membranes.

2.7 Conclusion

One should be very careful in extrapolating findings of C6C1 pathways in a plant e.g. *Arabidopsis* to other plants. It can not be excluded that particularly for secondary metabolites different localization and regulation of the pathways occurs in different plant species. Chorismate is biosynthesized in plastids, where also most of the enzymes discussed are localized. But chorismate may be transported out of plastids and further converted in other compartments. For example, plants overexpressing microbial SA genes without plastidial signal sequence still produced small amounts of SA, thus requiring transport of chorismate. AS has also been proposed to have a plastidial and a cytosolic form, though evidence is lacking. The flux through the different branches is quite different with the chorismate mutase (CM) pathway generally being the most active. Unraveling all the C6C1 pathways on the level of genes, proteins and intermediates including localization (transport) and regulation will be a major challenge for the coming years.