The evolution of chemical diversity in plants : pyrrolizidine alkaloids and cytochrome P450s in Jacobaea

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Chapter 6

Summary and conclusions

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Plants produce an astonishing variety of secondary metabolites (SMs) which are thought to play vital roles in the fitness of plants through ecological interactions (Wink, 2003; Moore *et al*., 2014). SMs enable plants to deal with antagonists (e.g. herbivores, pathogens, neighboring plants) and mutualists (e.g. pollinators, predators/parasitoids against herbivores, mycorrhizal fungi, rhizobium and other beneficial bacteria) as well as abiotic factors (e.g. UV light, drought, frost) (Kessler and Halitschke, 2007). The most characteristic features of SMs are their striking chemical diversity and inter- or intraspecific variation. So far, an estimated 200,000 SMs have been isolated and identified from plants (Kessler and Kalske, 2018). SMs are assigned to different compound classes such as glucosinolates, alkaloids, terpernoids and flavonoids. Within each chemical class, both qualitative and quantitative SM diversity are common in plants. For instance, in *Arabidopsis thaliana* 34 different glucosinolates have been identified, showing 20-fold difference in total concentration in leaves of different ecotypes (Kliebenstein *et al.*, 2001). As yet, it is poorly understood how SM diversity comes about and why it is maintained in nature (Moore *et al.*, 2014).

In this thesis we focus on SMs involved in plant protection against herbivores. Aiming at a better understanding of mechanisms behind SM diversity, researchers have put much effort in studying the distribution patterns of SMs under particular phylogenetic frameworks (Wink, 2003; Wink, 2008; Pelser *et al.*, 2005; Mint Evolutionary Genomics Consortium, 2018). At the level of a particular class of compounds (e.g. quinolizidine alkaloids, glucosinolates) there is a strong phylogenetic signal although some exceptions exist. This suggests that once the ability to produce the basic structures of these classes of compounds has evolved it is conserved through evolutionary time and not often convergent evolution takes place. In contrast the distributions of different chemical modifications of these structures on phylogenetic trees are often random. That is to say particular SMs within a class lack phylogenetic signals and phylogenetic distances are not correlated with differences in SM bouquets (Pelser *et al*., 2005; Mint Evolutionary Genomics Consortium, 2018; Chapter 2). This suggests that such modifications are not conserved and can rapidly appear or disappear on an evolutionary time scale. An evolutionary explanation would be that the basic structures that helps to protect plants against a broad set of herbivores comes about once and is conserved and that further evolutionary fine-tuning is dependent on the selective effects of the specific set of generalist and specialist herbivores each plant species is confronted with at a particular moment in evolutionary time. At the molecular level, the fact that new basic structures do not rapidly evolve in many plant families suggests that the enzymatic changes needed to create them are relatively complex. The further modification of newly evolved basic structures involves relative simple chemical reactions such as oxidation that are carried out by more general enzymes that are already present in the plant. The multiple functions of such an enzyme are subsequently reduced to single function by duplication of the underlying gene allowing optimizing the catalyzation for the new function (Hughes, 1994; DePristo, 2007). Examples

are the large gene families of cytochrome P450s (CYPs) of which the members show large similarity and that rapidly evolve (Bak *et al*., 2006; Frey *et al*., 2009; Chapter 3). Apparently this enables fast evolution, loss of phylogentic signal and rapid evolutionary fine-tuning of their efficacy. The process of evolutionary tinkering is most likely in addition speeded up by genetic changes in the expression of CYPs. The investigation of the diversity of both SM profiles and the CYP family in plants may allow insights into the evolution of SM pathways that coordinate the respective enzymes.

The pyrrolizidine alkaloids (PAs) of *Jacobaea* were used as the model system in this thesis. PAs are a class of SMs with typical great diversity that are constitutively formed in plants containing them and are thought to mediate the interactions between plants and herbivores (Hartmann, 1999). The *Jacobaea* species (26 species and formerly a part of *Senecio* species, Asteraceae) all produce PAs (Pelser *et al*., 2005; Langel *et al*., 2011) but the composition and concentration are often species-specific (Soldaat *et al*., 1996; Hartmann and Dierich, 1998; Langel *et al*., 2011). Nevertheless, individual PAs seem to have random distributions on the phylogenetic tree lacking phylogenetic signals (Pelser *et al*., 2005). Two hypotheses can be used to explain the random occurrences of PAs in *Jacobaea* species, i.e. (i) convergent evolution where the ability of plant species to produce particular PAs evolved several times, (ii) differential gene regulation of the PA pathway where all the plant species possess the machinery to produce those PAs but do not all express them. In order to figure out which hypothesis is correct, the occurrence and expression of genes underlying SM pathways need to be investigated.

So far, more than 400 PAs have been found (Chou and Fu, 2006), of which more than 100 PAs are macrocyclic senecionine-type (Hartmann and Witte, 1995; Langel *et al*., 2011). Most of our current knowledge of PA biosynthetic diversification has come from the studies of the 12-membered macrocyclic senecionine-type PAs in the Senecioneae (Langel *et al*., 2011). The primary product senecionine *N*-oxide synthesized in roots (Hartmann and Toppel, 1987; Hartmann *et al*., 1988; Hartmann *et al*., 1989) undergoes structural transformations in a position-specific and stereoselective manner resulting in the rearrangement of the skeletal structure and oxidative modifications thereof in shoots (Hartmann and Dierich, 1998; Pelser *et al*., 2005). The enzymes responsible for these processes have not been identified. CYPs catalyze a wide range of regiospecific, stereospecific and irreversible oxidation steps in plant SM biosynthesis (Renault *et al*., 2014), thus playing an important role in the evolution of chemical diversity. Given the common oxygenated site-specific modifications of PAs derived from the primary PA senecionine *N*-oxide, CYPs are good candidates for involvement in PA biosynthesis.

In this thesis the following questions were proposed: what are the distribution patterns of PAs in *Jacobaea* species and are these patterns related to their phylogenetic relationships? How does this PA diversity come about? Are CYPs involved in PA biosynthesis? Following these questions, the experimental chapters of this thesis can be divided into two sections: (i)

the evolution of PA diversity among and within *Jacobaea* species (Chapter 2), (ii) a candidate gene approach targeting CYPs for involvement in PA diversity (Chapter 3-5).

1. The evolution of PA diversity among and within *Jacobaea* species

In chapter 2, aiming to understand the mechanism behind chemical diversity, PA patterns of *Jacobaea* species were studied in a phylogenetic context. The presences and concentrations of 80 PAs in eight to ten week old leaves of 17 *Jacobaea* species including different individuals and populations grown in a controlled chamber were analyzed by LC-MS/MS. A great diversity of PA profiles was observed with the numbers of PAs ranging from 21 to 59 per species, and with the total PA concentrations ranging from 32.9 to 3835.7 µg/g dry weight. These profiles were largely confirmed to be species-specific both qualitatively and quantitatively, which is in line with previous findings (Soldaat *et al*., 1996; Hartmann and Dierich, 1998; Langel *et al*., 2011). Jacobine-like PAs, senecionine-like PAs and otosenine-like PAs contributed more to the classification of different *Jacobaea* species than other PA structural groups. PA patterns from different populations within some *Jacobaea* species (e.g. *J. alpina* and *J. paludosa*) differed from each other even surpassing differences between species. The phylogeny of 17 *Jacobaea* species were reconstructed using 11 chloroplast regions and three nuclear DNA genes to trace the evolution of PA diversity at species level. With ancestral state reconstruction of the occurrences of individual PAs, complex evolutionary patterns for almost all PAs were found showing that the occurrence of virtually all PAs evolved several times, which is in agreement with the findings of Pelser *et al*. (2005). Two different measures, Blomberg's *K* (Blomberg, 2003) and Pagel's λ (Pagel, 1999), were used to evaluate the correlations between quantitative PA traits and phylogenetic relationships, and significant phylogenetic signals for nine out of 80 PAs only under λ statistics were found. Given the common intraspecific PA diversity found in *Jacobaea* species (this thesis; Witte *et al*., 1992; Macel *et al*., 2004), it can be assumed that this high PA diversity is due to the regulation of PA biosynthesis genes in plants as a life strategy to meet their different biological needs rather than the evolutionary gains and losses of particular PA biosynthesis genes.

2. A candidate gene approach targeting CYPs for involvement in PA diversity

Testing our hypothesis that PA diversity in *Jacobaea* species is the result of the regulation of PA biosynthesis genes requires the genes underlying PA biosynthesis. CYPs have formed a large gene family in plants and have often been found to be involved in SM pathways as oxidative enzymes. As diversification of PAs is often through site-specific oxidation (Hartmann and Dierich, 1998; Pelser *et al*., 2005), CYPs are likely to be involved in PA biosynthesis. Yet, no public CYP database of *Jacobaea* species is available at the start of the studies described in this thesis. Therefore, a systematical study of CYPs was performed with regard to their diversity and evolution in *J. vulgaris* and *J. aquatica* as described in Chapter 3. In total, 221 (classified into eight clans and 38 families) and 157 (classified into eight clans and 35 families) full-length CYPs were retrieved from *de novo* assembled transcriptomes of *J.*

vulgaris and *J. aquatica*, respectively. Based on KEGG annotation, the CYPs assigned as being SM metabolic pathway enzymes were all from the CYP71 clan but no CYPs were assigned as being involved in alkaloid pathways. This does not necessarily mean that they are not involved in alkaloid biosynthesis since the current KEGG database does not contain information about PA biosynthetic enzymes. Phylogenetic analyses of the six largest CYP families (CYP71, CYP76, CYP706, CYP82, CYP93 and CYP72) from the two *Jacobaea* species, two other members of the Asteraceae, *Helianthus annuus* and *Lactuca sativa* and the outgroup of *Arabidopsis thaliana* were performed. The phylogenetic trees showed strong lineage-specific expansion of CYPs, suggesting that the evolution of CYPs has been very fast even within the Asteraceae family. Only CYPs of the closely related species *J. vulgaris* and *J. aquatica* were found often in pairs, confirming a close relationship in evolutionary history of these two species. The studies described in Chapter 3 provide a CYP database for future exploration of their functions, including possible involvement in PA biosynthesis and PA diversity.

Chapter 4 describes an attempt to identify candidate CYPs that may be involved in PA biosynthesis based on the association between metabolic and transcriptomic profiles between different *Jacobaea* samples grown under controlled conditions. *J. aquatica* and four groups of F_2 hybrids of a cross between *J. vulgaris* and *J. aquatica* with PA contrasts were used for constitutive PA contrasts, especially in jacobine-like and erucifoline-like PAs. A methyl jasmonate treatment on tissue culture plants of *J. vulgaris* were performed to induce increase of erucifoline-like PAs. In total, 44 PAs were detected by LC-MS/MS and PA profiles of different *Jacobaea* samples were compared separately in the constitutive and induced groups by summing up concentrations of PAs containing the same site-specific oxidative modifications which might be catalyzed by a CYP enzyme: 15,20-epoxidation, 12,13-epoxidation or 19-hydroxylation, 18-hydroxylation, 13,19-dehydrogenation and 8-oxidation. RNA sequencing was performed separately for the constitutive and induced groups to analyze the expression of CYPs which may be involved in PA oxidative conversions. In total, 33 and 27 CYP candidate genes were sieved out for the constitutive and induced PA conversions, respectively. Most of these candidate enzymes were from the CYP71 clan without known functions. There were 11 CYP subfamilies found both in the constitutive and induced groups, where three subfamilies (CYP72A, CYP706E, CYP82Q) may be responsible for the formation of erucifoline-like PAs which contain both 12,13-epoxidation and 19-hydroxylation.

Chapter 5 describes functional tests of eight CYP candidates for involvement in the PA biosynthesis pathway using heterologous expression in yeast and *in vitro* enzyme assays using microsomal membrane preparations presumably containing the expressed CYPs. None of the eight CYP enzyme preparations showed PA conversion with the selected PA substrates (senecionine/integerrimine, seneciphylline, jacobine, erucifoline or a PA mixture as well as the respective *N*-oxides) based on *in vitro* enzyme assays using extracted microsomal membranes. The reasons of the negative results might be lack of efficient expression, and/or requirement for more optimal reaction conditions or better detection method. More likely is

that the tested eight CYPs are not enzymes responsible for PA biosynthesis, or at least do not act on the tested substrates.

3. Discussion and conclusions

Jacobaea species have been frequently used to study their PA diversity (Vrieling *et al*., 1993; Hartmann and Dierich, 1998; Macel *et al*., 2004; Cheng *et al*., 2011). Compared with previous studies, we studied both qualitative and quantitative PA variation at more levels including among species, among populations and among individuals at the same time. Our results revealed stronger evidence that PA patterns of *Jacobaea* species are indeed species-specific both in concentrations and compositions, though for some species PA profiles also differed largely between populations. It is the first study showing that the distribution pattern of concentrations among and within *Jacobaea* species was highly similar to that of compositions, implying the same or closely related mechanisms behind quantitative and qualitative variation of PAs.

The occurrence of individual PAs was studied at species level and limited phylogenetic signals were found. A reasonable assumption is that all *Jacobaea* species possess the machinery for PA production and that differences in PA occurrence are due to differential gene regulation. Based on the fact that PA diversity is largely created by oxidation creations, CYPs, a major class of oxidative enzymes in plants, were chosen as an entry for identification of PA biosynthesis genes. Based on current scientific literature, the curated CYP database developed in this thesis is the first in *Jacobaea* species. A gene-to-metabolite approach was used to identify CYP candidates possibly involved in PA biosynthesis and the functions of eight candidate CYP enzymes was checked but this did not lead to any indication for their involvement in PA biosynthesis. The majority of candidate gene identified in Chapter 4 still needs to be tested. There is a possibility that the enzymes underlying PA biosynthesis are not CYPs but other oxidative enzymes such as flavin-dependent monooxygenases or peroxidases (Burton, 2003), for which gene-to-metabolite correlations can also be retrieved from the data described in Chapter 4.

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