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

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# Chapter 1

General introduction

# General introduction

Secondary metabolites (SMs) are ubiquitous in the plant kingdom. So far, an estimated 200,000 SMs have been isolated and identified from plants (Kessler and Kalske, 2018). The total number of SMs in plants is estimated to be more than 500,000, so many more SMs await to be discovered (Hadacek, 2002). Often a distinction is made between primary metabolites which are directly involved in plants' growth, development and reproduction and SMs. The latter, also referred to as small molecules are known to play a role in a plant's defense against abiotic and biotic stresses although for many of SMs the function is still unknown (Fig. 1; Hartmann, 1996; 1999). Plant SMs function as defense compounds deterring herbivores and pathogens, and as signaling compounds attracting pollinators, predators and parasitoids against herbivores and mediating interactions with mycorrhizal fungi and beneficial bacterial and with neighboring plants (Kessler and Halitschke, 2007). In addition, SMs also protect plants against abiotic stressors such as UV light, drought and frost (Isah, 2019). Due to the large number, high structural diversity and multifunctionality of SMs, it is still an ongoing challenge to understand how this SM diversity comes about, and why such a large diversity is maintained in nature (Moore *et al.*, 2014; Kessler and Kalske, 2018).



In this thesis this question was studied using the pyrrolizidine alkaloids (PAs) of *Jacobaea* species as the study system from an evolutionary and biosynthetic perspective.

### 1. Secondary metabolite (SMs)

1.1 SM diversity

1.1.1 SM diversity among chemical classes

The isolated and identified SMs have been assigned into different chemical classes (Fig. 1; Hartmann, 1996). The SMs containing nitrogen are classified as glucosinolates, cyanogenic glycosides or alkaloids. Chemical classes of SMs without nitrogen contain amongst others terpenes, polyketides, flavonoids, quinones, coumarins and tannins. These chemical classes are further divided into subgroups. For example, alkaloids are one of the most diverse compound classes with approximately over 25,000 structures found in higher plants. They are usually classified according to the nature of basic chemical structures. Following this system, heterocyclic alkaloids are further subdivided into groups such as isoquinoline, indole, quinolone, quinazoline, pyrrolizidine, and tropane alkaloids. (Funayama and Cordell, 2015).

1.1.2 SM diversity within chemical classes

SM diversity within compound classes is very common. Each class of SMs mostly possesses a number of similar molecules showing the same skeleton while differing in substitution groups by adding a number of polar and non-polar substituents. This structural diversity is well documented by abundant examples. For instance, 34 glucosinolates were reported in *Arabidopsis thaliana* (Kliebenstein *et al.*, 2001), which are molecules consisting of a *β*-

thioglucose moiety, a sulfonated oxime, and a variable side chain derived from various amino acids. Thirty-seven structurally related PAs have been detected in *Jacobaea vulgaris*, *Jacobaea aquatica* and their hybrids (Cheng *et al.*, 2011a), where most PAs are assumed to be derived from senecionine *N*-oxide with minor chemical modifications (Hartmann and Dierich, 1998; Pelser *et al.*, 2005). Apart from the structural diversity, SMs often show remarkable quantitative variation. This is well exemplified by the abovementioned glucosinolates which showed about 20-fold difference in total concentrations among leaves of different ecotypes (Kliebenstein *et al.*, 2001).



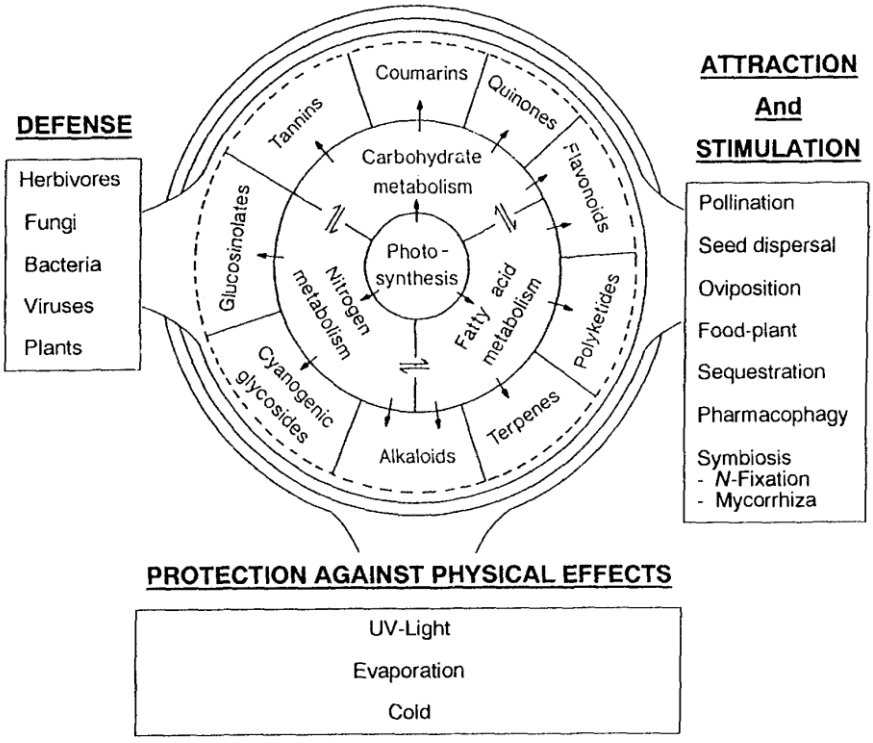


**Figure 1.** Classification and ecological functions of plant secondary metabolites (from Hartmann, 1996).

## 1.2 Distribution patterns of SMs

### 1.2.1 Distribution patterns of particular chemical classes

Some classes of SMs have a wide distribution among members of different plant phyla. For example, terpenes (isoprenes) responsible for plants' fragrance comprise one of the most diverse compound classes (Kessler and Kalske, 2018). Flavonoids comprise over 6000 different chemical moieties found in virtually all plants and fruits (Havsteen, 2002; Cvorovi *et al.*, 2018). Nonetheless, most classes of SMs are restricted to specific plants or plant lineages

(Ober, 2010), implying a strong phylogenetic signal although some exceptions can be observed. For instance, glucosinolates are major SMs near-universally in Brassicaceae, 1Capparidaeae and Caricaceae (Moore *et al.*, 2014), and benzylisoquinoline alkaloids occur mainly in the Papaveraceae, the Ranunculaceae, the Berberidaceae and the Menispermaceae (Ziegler and Facchini, 2008), while PAs distribute preferably in the Asteraceae, the Boraginaceae, the Fabaceae and the Orchidaceae families (Hartmann and Witte, 1995; Langel *et al.*, 2011).

1.2.2 Distribution patterns of SMs within a particular class



Commonly within families or clades, the sort of SM class used by plants tends to be conserved so that most genera and species produce chemicals that fall into a particular conserved class. However, the presence or absence of a particular SM within a particular class in phylogenetically related taxa is commonplace (Pelser *et al.*, 2005; Mint Evolutionary Genomics Consortium, 2018). In another words, the distribution of different chemical modifications of the basic structures on phylogenetic trees are often random. For example, the presence of monoterpenes exhibited a random distribution across the phylogeny of Lamiaceae, where 14 out of 57 showed significant phylogenetic signals (Mint Evolutionary Genomics Consortium, 2018). Whether this patchy distribution of SMs is the result of convergent evolution or of differential gene regulation is still open to debate.

1.3 Biochemical roots of plant SM diversity

With the development of biochemistry and molecular biology, questions about how such high SM diversity come about is partially answered. Scientists have summarized five hypotheses regarding to biochemical processes explaining the mechanisms underlying biosynthetic diversity and unique SM bouquets among and within plant individuals and species (reviewed by Kessler and Kalske, 2018): (i) simple chemical precursors originating from primary metabolism enable many possible combinations based on those subunits, (ii) a great diversity of genes are encoding a similar type of functional enzymes, catalyzing a diverse bouquet of SMs from common precursors, (iii) some multifunctional biosynthetic enzymes can generate a number of products out of the same precursor as a plastic system, (iv) the low substrate specificity and rapid functional divergence of modifying enzymes that are from large gene families, (v) spatially and temporally differential gene expression of biosynthesis genes can influence SM diversity of a plant quantitatively and qualitatively. These five mechanisms may contribute to SM diversity individually or additively.

1.3 Hypotheses explaining plant SM diversity

SMs are essential for plants' survival and reproductive fitness. SMs are adaptive traits that are subject to natural selection during evolution (Wink, 2003). Although how and why chemical diversity is maintained over evolutionary time is still not well understood, several hypotheses have been put forward to explain SM diversity. The screening hypothesis (Jones and Firn, 1991; Firn and Jones, 2003; 2009) states that the likelihood of producing new bioactive

compounds is enhanced with a great diversity of mostly inactive and inexpensive compounds. This hypothesis states that SMs arise via mutation with an inherently low probability of possessing any biological activity. However, genes encoding certain SM biosynthetic pathways are found to be grouped in gene clusters within plant genomes, which suggests strong selection in driving functional modularity of SM biosynthesis that is not derived from a simple breakdown process (Nützmann *et al.*, 2016). The "evolutionary arms race" between plants and herbivores is also a widely known theory (Ehrlich and Raven, 1964). Plants can escape from herbivory by evolving novel defense compounds, entering a new adaptive zone. Conversely, herbivores adapted to the novel SMs gain greater fitness and reciprocal evolutionary changes occur subsequently in the plants. Here we can find a striking analogy with mankind being in an evolutionary arms race with specialist herbivores to protect our crops which requires the continuous development of new and more diverse insecticides because the herbivores rapidly develop resistance. The idea of coevolution between phytochemicals and herbivores is broadly accepted (Futuyma and Agrawal, 2009; Maron *et al.*, 2019). Berenbaum and Zangerl (1996) proposed the "interaction diversity" hypothesis to explain SM diversity. It is assumed that different SMs have different biological functions. Plant SM diversity is beneficial in a diverse community and thus is the ecological consequence of the plant's interaction with diverse biotic and abiotic environments. This hypothesis is in line with the findings that a more diverse and more specialized community of herbivores is associated with an elevated chemical diversity of *Piper* species (Richards *et al.*, 2015). SM diversity is explained by synergism, where the effectiveness of a defense compound increases in the presence of another (Berenbaum *et al.*, 1991; Rasmann and Agrawal, 2009). Synergistic effects of plant SMs have been demonstrated in a number of cases (Dyer *et al.* 2003; Leckie *et al.*, 2016; Liu *et al.*, 2017).



**2. Pyrrolizidine alkaloids (PAs)**

In this thesis, we focus on SMs involved in plant protection against herbivores. PAs comprise a typical class of SMs that are constitutively formed in plants containing them and are thought to mediate the interactions between plants and herbivores (Hartmann, 1999). PAs are ester alkaloids containing a necine base which is esterified with one or more necic acids, forming monoesters, open-chain diesters or even triesters and macrocyclic diesters. Chemical modifications of both the necine base and the necic acid moiety result into a large number of PA structures found in plants (Fig. 2; Hartmann and Witte, 1995). PAs occur as both tertiary amines (free bases) and *N*-oxides (Fig. 2; Joosten *et al.*, 2011; Patrick *et al.*, 2018), where the polar salt-like *N*-oxide form is more common. Thus far, more than 400 PAs have been found in over 6,000 plant species (Chou and Fu, 2006). Of these ca. 6,000 angiosperm species in distantly related families more than 95% belong to four families, i.e. the Asteraceae (the tribes Senecioneae and Eupatorieae), the Boraginaceae (most genera), the Fabaceae (mainly the genus *Crotalaria*) and the Orchidaceae (ca. 40 species) (Hartmann and Witte, 1995; Langel *et al.*, 2011). Within Senecioneae, approximately 190 PAs have been detected in circa 300 species, of which more than 100 PAs are of the macrocyclic senecionine-type containing four

structural groups, i.e. the senecionine group, the senecivernine group, the nemorensine group and the rosmarinine group (Fig. 3; Hartmann and Witte, 1995; Langel *et al.*, 2011). The senecionine group were further subdivided into different structural subgroups based on their structural characteristics, including senecionine-like PAs, jacobine-like PAs, erucifoline-like PAs and otosenine-like PAs (Pelser *et al.*, 2005; Cheng *et al.*, 2011).



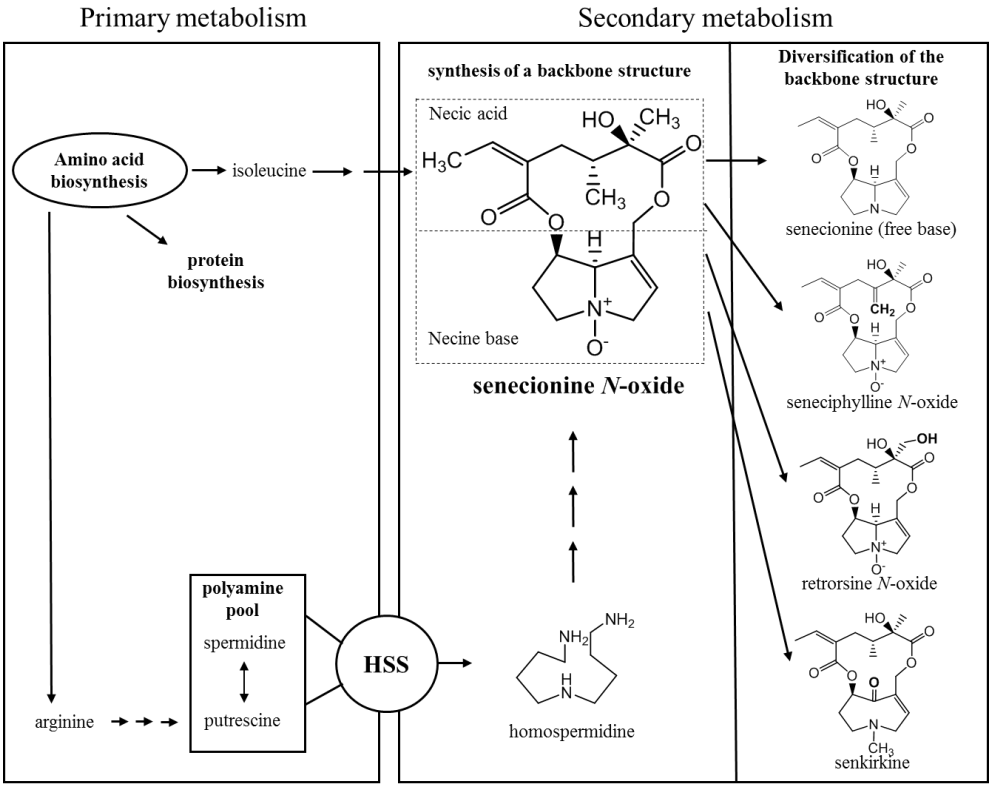


**Figure 2.** Simplified scheme of PA biosynthesis in *Senecio* and *Jacobaea* (from Langel *et al.*, 2011 with minor modification).

2.1 PA biosynthesis, translocation and accumulation in *Senecio* and *Jacobaea* species

*Senecio* and *Jacobaea* species of the tribe Senecioneae have been often used to study PA biosynthesis (Hartmann and Toppel, 1987; Toppel *et al.*, 1987; Hartmann *et al.*, 1989; Sander and Hartmann, 1989; Hartmann and Dierich, 1998). Earlier $^{14}$C-labelled tracer studies revealed that PA biosynthesis is closely related to polyamine metabolism (Hartmann and Toppel, 1987; Hartmann *et al.*, 1988), where arginine is converted successively to putrescine and spermidine which are the two substrates for the formation of homospermidine (Fig. 2; Böttcher *et al.*, 1994), the first specific intermediate in the PA biosynthesis pathway (Böttcher *et al.*, 1993). The enzyme responsible for this conversion, namely homospermidine synthase (HSS), is the

only PA pathway-specific enzyme that has been identified so far (Böttcher *et al.*, 1993; Böttcher *et al.*, 1994; Langel *et al.*, 2011). It has been suggested that the HSS encoding gene originated from the gene encoding deoxyhypusine synthase by means of gene duplication and diversification based on similarities in their sequences and biochemical reactions (Ober and Hartmann, 1999). Homospermidine is exclusively incorporated into the necine base moiety of PAs without any turnover (Fig. 2; Böttcher *et al.*, 1993). Senecionine *N*-oxide (Fig. 2) is found as the first alkaloid of PA biosynthesis which is synthesized only in roots of *Senecio* and *Jacobaea* species (Hartmann and Toppel, 1987; Toppel *et al.*, 1987; Hartmann *et al.*, 1989; Hartmann and Dierich, 1998). It is not known how homospermidine is converted to senecionine *N*-oxide.



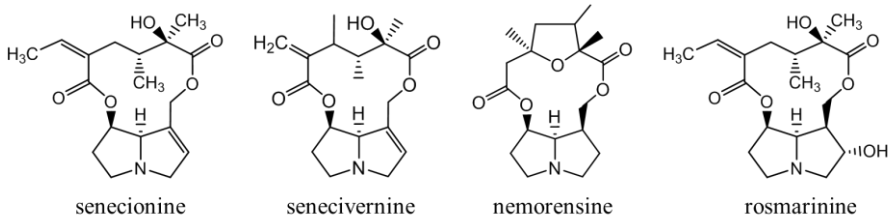


**Figure 3.** Pyrrolizidine alkaloids of the macrocyclic senecionine-type encompassing the senecionine group (12-membered), the senecivernine group (12-membered), the nemorensine group (13-membered) and the rosmarinine group (12-membered).

In *Senecio* and *Jacobaea* plants that were studied, senecionine *N*-oxide synthesized in roots is via the phloem allocated all over the plant and is accumulated at the preferential storage sites, i.e. inflorescences and peripheral tissues of leaves and stems (Hartmann *et al.*, 1989; Witte *et al.*, 1990). Phloem loading and unloading of polar salt-like PA *N*-oxides is predicted to be carrier-mediated (Ehmke *et al.*, 1987; 1988) since species which do not produce PAs were shown to be unable to translocate PAs via the phloem (Hartmann *et al.*, 1989). In shoot organs, the backbone structure senecionine *N*-oxide is transformed into structurally related PAs (Fig. 2; Fig. 4) without any turnover or degradation (Sander and Hartmann, 1989; Hartmann and Dierich, 1998). PAs are assumed to be selectively taken up and stored in cell vacuoles by a specific *N*-oxide carrier (Ehmke *et al.*, 1987, 1988). Consequently, *Senecio* and *Jacobaea* species have qualitative and quantitative PA profiles resulting from several interacting processes, i.e. (i) *de novo* synthesis of senecionine *N*-oxide in roots, (ii) dynamic long-distance translocation of senecionine *N*-oxide into shoots, (iii) structural diversification of senecionine *N*-oxide with varying efficiency between different organs, (iv) continuous allocation of PAs in the plant, (v) selective uptake and storage of PAs in vacuoles (Hartmann and Dierich, 1998).

### 2.2 PA diversification

Most of our current knowledge of PA biosynthetic diversification has come from the studies of the 12-membered macrocyclic senecionine-type PAs (Fig. 3) in the Senecioneae (Langel *et*

*al.*, 2011). Senecionine *N*-oxide synthesized in roots has been identified as the backbone structure of PA conversion by label tracer studies (Hartmann and Toppel, 1987; Hartmann *et al.*, 1988; Hartmann *et al.*, 1989; Hartmann and Dierich, 1998). To a limited extent in roots senecionine *N*-oxide may be converted to structurally related derivatives such as seneciphylline *N*-oxide (Toppel et a., 1987; Sander and Hartmann, 1989). However, shoots have been identified as the major sites for PA transformations in *Senecio* and *Jacobaea* species (Hartmann and Dierich, 1998). Tracer experiments revealed that senecionine *N*-oxide undergoes structural transformations in a position-specific and stereoselective manner resulting in species-specific PA bouquets (Fig. 4; Hartmann and Dierich, 1998). With the exception of senecivernine transformations between PAs can be explained by two main reactions (i.e. conversion of retronecine to otonesine and site-specific epoxidation) and simple one- or two-step reactions (20-*Z*/*E*-isomerization, 13,19-dehydroxylation, site-specific hydroxylation, hydrolysis of 15,20-epoxide, chlorolysis of 15,20-epoxide, site-specific *O*-acetylation and *N*-deoxidation; Pelser *et al.*, 2005). This suggests that PA diversification is a highly plastic process (Hartmann and Dierich, 1998). The enzymes underlying these elaborate steps of chemical modifications have not been identified.

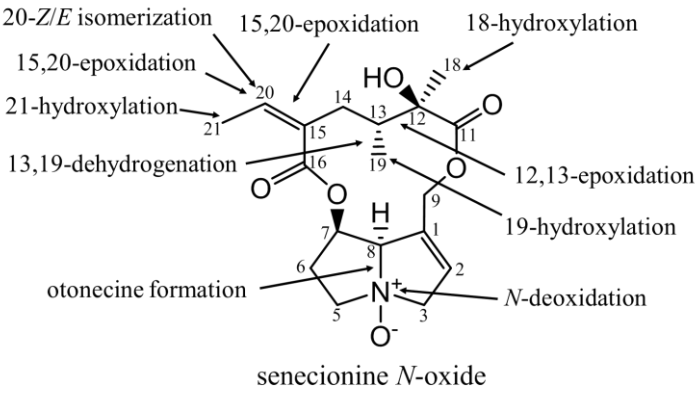


**Figure 4**. Structural modifications of senecionine *N*-oxide in position-specific and stereoselective manners.

## 2.3 PA diversity in *Senecio* and *Jacobaea* species

### 2.3.1 Interspecific diversity

A great diversity of PA patterns have been found in *Senecio* and *Jacobaea* species (Langel *et al.*, 2011). PA profiles are often species-specific (Hartmann and Dierich, 1998), but phylogenetic relationships are not correlated with PA composition at the level of individual PAs (Langel *et al.*, 2011). The 26 species of the genus *Jacobaea* (formerly a section of the genus *Senecio*) all produce PAs (Pelser *et al.*, 2005; Langel *et al.*, 2011) but their PA profiles

are believed to be species-specific (Soldaat *et al.*, 1996; Hartmann and Dierich, 1998; Langel *et al.*, 2011). PAs appear to be incidentally distributed over *Jacobeae* species, demonstrating little phylogenetic signal. Even phylogenetically close species can differ extensively in their PA compositions (Pelser *et al.*, 2005). For example, PA profiles of *J. vulgaris* and *J. aquatica* are generally different qualitatively and quantitatively (Pelser *et al.*, 2005; Langel *et al.*, 2011; Cheng *et al.*, 2011a), although these two species are closely related (Pelser *et al.*, 2004).

2.3.2 Intraspecific diversity

Besides interspecific variation, a large variety of PA profiles within species were abundantly found. Population-level studies revealed that different populations within species often show considerable differences in their PA compositions and concentrations (Witte *et al.*, 1992; Macel *et al.*, 2004; Joshi and Vrieling, 2005). This intraspecific diversity is well documented by different chemotypes of *J. vulgaris* and *J. erucifolia*. By evaluating populations originating from different geographic locations, 'jacobine chemotype' and 'erucifoline chemotype' were found for *J. vulgaris*, while 'erucifoline chemotype' and 'eruciflorine chemotype' were found for *J. erucifolia* (Witte *et al.*, 1992). More chemotypes were found for *J. vulgaris* in a later study conducted by Macel *et al.* (2004), in which they distinguished senecionine, jacobine, erucifoline and mixed chemotypes. In addition to the difference between populations, variation in PA composition between individual plants within populations is also considerable. The relative abundances of jacobine in 412 plants from half-sib families of the population collected from Meijendel (The Netherlands) varied from 41 to 100% of total PA, whereas that of erucifoline ranged from 0 to 19% (Macel *et al.*, 2004).

2.3 Driving factors of PA variation

2.3.1 Genetic bases

Both PA concentration and composition are under genetic control. As aforementioned, HSS is so far the only enzyme identified that is involved in PA biosynthesis. It is believed that the total PA concentration of a plant is closely related to HSS as it catalyzes the formation of the first PA intermediate, homospermidine, and PAs do not undergo turnover except for chemical diversification (Ober and Hartmann, 1999). It was indicated that under controlled conditions 50-100% of the total variation in PA concentration of *J. vulgaris* is due to genetic differences in a diallel cross and a half-sib analysis (Vrieling *et al.*, 1993). The comparison of PA profiles between 10 clonal families of *J. vulgaris* from different populations showed that the variation in PA composition within clonal families was smaller than that among these families, implying that PA composition is genetically determined (Macel *et al.*, 2004). The finding that senecionine *N*-oxide is transformed into unique PAs in different *Senecio* and *Jacobeae* species clearly indicates that species-specific PA profiles are brought about by genetically controlled specific processes (Hartmann and Dierich, 1998). It is not clear whether quantitative and qualitative variations of PAs are controlled by the same genetic mechanism or distinct mechanisms.

### 2.3.2 Environmental factors

Vrieling *et al.* (1993) suggested that PA profiles in populations of *J. vulgaris* are under natural selection. The potential roles of herbivores in PA variation as selective forces have been extensively investigated (Vrieling and de Boer, 1999; Macel *et al.*, 2002; Joshi and Vrieling, 2005; Cheng *et al.*, 2011b; Kostenko *et al.*, 2012; Cheng *et al.*, 2013). It was hypothesized that a complex PA profile with high structural diversity among and within species forms a powerful defense barrier which cannot be easily overcome by generalist herbivores since they need to cope with numerous chemical structures (Hartmann and Dierich, 1998; Pelser *et al.*, 2005). In contrast specialist herbivores are attracted by PAs. The cinnabar moth, a specialist herbivore causing the majority of leaf loss in *J. vulgaris* preferred to oviposit on plants with more tertiary amines of the jacobine-like and otosenine-like PAs (Cheng *et al.*, 2013). In addition, both the cinnabar moth and the garden tiger moth sequester PAs in their bodies (Aplin and Rothschild, 1972). These findings indicate that specialist herbivores too may potentially act as a selective force on variation in PA composition and concentration. Besides herbivores, soil-borne microorganisms also affect PA composition of *J. vulgaris* (Joosten *et al.*, 2009).

PA patterns in plants are also affected by abiotic factors such as nutrients, water and light. PA concentrations in the shoots and roots of *J. vulgaris*, *J. aquatica* and *S. vulgaris* were significantly reduced with increasing nutrient supplies. This decreased PA concentration was assumed to be due to the dilution effect from increased biomass following high nutrient supplies (Hol *et al.*, 2003; Hol, 2011). Conversely, *J. vulgaris* plants grown under nutrient stress tend to have higher PA concentrations compared with those grown under normal conditions (Vrieling and van Wijk, 1994). Water availability and light intensity are two other factors which affect PA concentrations. PA concentrations of *J. vulgaris* differed significantly under drought-stressed (Vrieling *et al.*, 1993) and light-limited conditions (Vrieling and van Wijk, 1994) compared to controls.

## 3. Cytochrome P450s (CYPs)

Genes involved in SM biosynthesis have often evolved from genes involved in primary metabolism by gene duplication with successive diversification (Ober, 2010; Moore *et al.*, 2014). Many of these genes involved in SM pathways belong to large gene families (Kessler and Kalske, 2018), such as cytochrome P450s (Bak *et al.*, 2006; Frey *et al.*, 2009). CYP genes form a large family in any given plant species (Mizutani, 2012). CYPs catalyze a wide range of regiospecific, stereospecific and irreversible steps in plant SM biosynthesis (Renault *et al.*, 2014), thus playing an important role in the evolution of chemical diversity. CYP enzymes are a major class of oxidative enzymes in eukaryotes. Given the oxidative transformations from the primary PA senecionine *N*-oxide to derived PAs, CYPs are possible candidates for performing these oxidations. Although amino acid sequences of CYP proteins are diverse, their overall topology and structural fold have remained conserved during evolution (Werck-Reichhart and Feyereisen, 2000; Bak *et al.*, 2011). CYP proteins have a number of conserved

motifs (Fig. 5; Bak *et al.*, 2011; personal communication of Prof. Dr. David R. Nelson) which make the recognition of CYPs in novel non-annotated transcriptomes possible.

### 3.1 Motifs of CYP proteins

The motifs of CYPs are few in the N-terminal region and more abundant in the C-terminus (Fig. 5; Nelson, 2004). CYPs all share a highly conserved catalytic center, where heme with iron is coordinated to the thiolate of a cysteine that is conserved in all CYP sequences. The heme-binding signature is located about 50 aa from the C-terminus. Upstream from the heme-binding domain is the PERF/W motif followed by a small PKG motif in a distance of ca. 20 aa. N-terminal of the PKG motif is the most conserved region in CYP proteins, the ExxR motif (K-helix). The E and R amino residues of the ExxR motif form a salt bridge with the R amino acid residue of the PERF/W motif, which is generally considered to be essential for the stability of the core structure (Hasemann *et al.*, 1995). This ERR triad is conserved in all plant CYP sequences. At about 200 aa from the C-terminus, I-helix (AGx[E,D]T) is regarded as an oxygen-binding motif containing the conserved glycine pointing at the heme center and the conserved threonine pointing to oxygen binding site (Li *et al.*, 2008). While at the N-terminus there is a proline rich membrane hinge at about 30 aa, and often but not always a C-helix (Wxxx[R,K]) at about 130 aa. All plants described so far are bound to a membrane, usually the ER membrane, through a short hydrophobic segment at their N-terminus (Fig. 5; Bak *et al.*, 2011).

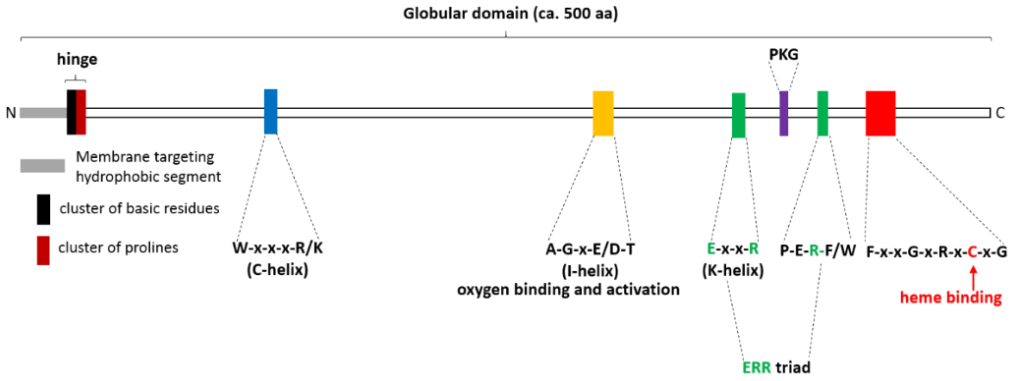


**Figure 5**. Conserved structures and sequences in classic CYP proteins..

### 3.2 Organization and evolution of CYPs in plants

CYPs are found in virtually all organisms, but the numbers have exploded in plants (Werck-Reichhart and Feyereisen, 2000). CYPs are classified and named following recommendations of a nomenclature committee (Nelson, 2009). So far, there are 127 CYP families in the plant kingdom (Nelson and Werck-Reichhart, 2011), of which the numbers of CYP families have plateaued at 59 in angiosperms (Hamberger and Bak, 2013). CYPs of land plants are grouped in 11 distinct clans according to sequence similarities. Furthermore, plant CYPs are divided

into A-type and non-A-type based on phylogenetic relationships. The A-type CYPs (CYP71 clan) form a monophyletic clade, whereas the non-A-type CYPs (the remaining 10 clans) are more diverse and do not form a coherent group in a phylogenetic sense (Hamberger and Bak, 2013). The A-type CYP71 clan is the youngest evolved clan as indicated by phylogenetic analyses of plant CYPs (Nelson and Werck-Reichhart, 2011; Bak *et al.*, 2011). This clan appears to have evolved via successive gene duplication events (Sezutsu *et al.*, 2013), leading to often species-specific expanded families (Hamberger and Bak, 2013). The CYP71 clan comprises more than half of all plant CYPs and consequently, harbors a great diversity of functions (Nelson and Werck-Reichhart, 2011). CYP72 and CYP85 are two other clans that have expanded dramatically in plants. It is more challenging to predict the functions or substrate preferences of members in the CYP71, CYP72 and CYP85 clans due to their dramatic expansion, even though phylogeny within families or subfamilies can serve as a guide to function prediction (Nelson and Werck-Reichhart, 2011).



3.3 Heterologous expression of plant CYPs

All plant CYPs are membrane-anchored by hydrophobic N-terminal regions, usually anchored on the cytoplasmic surface of the ER. They use molecular oxygen as the oxygen donor, which is coordinated by an iron atom in the heme prosthetic group. To be active, CYPs need to be coupled with electron-donating proteins, CYP reductases or cytochrome $b_5$, which are also anchored to the surface of the ER. Most commonly, via the NADPH-dependent CYP reductase (CPR) heme-bound $O_2$ is activated by the successive transfer of two electrons from NADPH (Bak *et al.*, 2011), leading to regiospecific and stereospecific oxidative attack of a plethora of substrates.

Heterologous expression of CYP proteins is an important step for their functional characterization. The heterologous expression of plant CYPs has mostly been performed in yeast, bacterial or insect cells for heterologous expression, with yeast being the most frequently used organism (Schuler and Werch-Reichhart, 2003; Duan and Schuler, 2006). There are several advantages for using the yeast *Saccharomyces cerevisiae* as heterologous expression host for plant CYPs, i.e. (i) the presence of an ER membrane environment and post-transcriptional modification systems, (ii) the availability of modified yeast strains expressing plant CPR genes (e.g. WAT11; Pompon *et al.*, 1996), (iii) the availability of vectors (e.g. pYeDP60) with high yielding galactose-inducible GAL10-CYC1 hybrid promoters (Urban *et al.*, 1990; Pompon *et al.*, 1996), (iv) relatively low costs, high efficiency and rapid growth (Pompon *et al.*, 1996; Hamann and Møller, 2007).

**4. Research questions**

The research described in this thesis aimed at understanding how PA diversity comes about from the perspectives of evolution and biosynthesis of PAs. In particular, the distribution patterns of PAs were studied in a phylogenetic context. CYPs were studied for involvement in PA biosynthesis and PA diversity. By using *Jacobaea* species as the model system, the following questions were addressed:

(i) Do *Jacobaea* species indeed have species-specific PA profiles at a larger scale including among species, among populations and among individuals with respect to both concentration and composition? Do distributions of individual PAs among *Jacobaea* species show phylogenetic signals?

(ii) How diverse are CYPs of *Jacobaea* species? What are evolutionary patterns of CYPs between two *Jacobaea* species, and two other members of the Asteraceae, *Helianthus annuus* and *Lactuca sativa*, and the outgroup *Arabidopsis thaliana*?

(iii) Are abundance patterns of PAs with site-specific oxidative modifications related to expression patterns of particular CYPs? Is it in this way possible to identify CYP candidates for specific oxidation steps?

(iv) Are identified candidate CYPs indeed involved in PA biosynthesis when functionally tested via heterologous expression in yeast and *in vitro* enzyme assays?



### 5. Outline of this thesis

In **Chapter 2**, both qualitative and quantitative PA variations for leaves of 17 *Jacobaea* species including different individuals and populations grown under controlled conditions were studied. Within the phylogenetic context of *Jacobaea* species, the evolutionary histories and phylogenetic signals of individual PAs were investigated in order to understand how PA diversity is related to species phylogeny.

CYPs often perform oxidative reactions in the biosynthesis of SMs and thus are crucial players in the evolution of chemical diversity. In **Chapter 3** the diversity and evolution of CYPs of *J. vulgaris* and *J. aquatica* were evaluated. The resulting database of CYPs was used for future exploration of their functions, including possible involvement in PA biosynthesis and PA diversity in later chapters.

In **Chapter 4**, PA profiles and expression profiles of CYPs of *J. vulgaris*, *J. aquatica*, and their hybrids with contrasting PA profiles were associated to discover candidate CYPs potentially involved in PA biosynthesis. Subsequently, in **Chapter 5** eight CYP candidates were tested using heterologous expression in yeast and *in vitro* enzyme assays.

Finally in **Chapter 6** the discussion and conclusions of the findings described in this thesis are presented.

### References

Aplin RT, Rothschild M. 1972. Poisonous alkaloids in the body tissue of the garden tiger moth (*Arctia caja* L.) and the cinnabar moth (*Tyria jacobaea* L.) (Lepidoptera). In: De Vries A, Kochva E (eds) Toxins of animal and plant origin. New York: Gordon & Breach Sci Pub Vol 2, pp.579-595.

Bak S, Beisson F, Bishop G, Hamberger B, Höfera R, Paquette S, Werck-Reichhart. 2011. Cytochrome P450. *The Arabidopsis Book* 9:e0144.

Berenbaum MR, Nitao JK, Zangerl AR. 1991. Adaptive significance of furanocoumarin diversity in *Pastinaca sativa* (Apicaceae). *Journal of Chemical Ecology* 17:207-215.

Berenbaum MR, Zangerl AR. 1996. Phytochemical diversity: adaptation or random variation. In: Remeo JT, Saunders JA, Barbosa P (eds) Phytochemical Diversity and Redundancy in Ecological Interactions. New York: Plenum pp.1-24.

Böttcher F, Adolph R-D, Hartmann T. 1993. Homospermidine synthase, the first pathway-specific enzyme in pyrrolizidine alkaloid biosynthesis. *Phytochemistry* 32:1373-1384.

Böttcher F, Ober D, Hartmann T. 1994. Biosynthesis of pyrrolizidine alkaloids: putrescine and spermidine are essential substrates of enzymatic homospermidine formation. *Canadian Journal of Chemistry* 72:80-85.



Chang A, Hartmann T. 1998. Solubilization and characterization of a senecionine *N*-oxygenase from *Crotalaria scassellatii* seedlings. *Phytochemistry* 49:1859-1866.

Cheng D, Kirk H, Mulder PPJ, Vrieling K, Klinkhamer PGL. 2011a. Pyrrolizidine alkaloid variation in shoots and roots of segregating hybrids between *Jacobaea vulgaris* and *Jacobaea aquatica*. *New Phytologist* 192:1010-1023.

Cheng D, Kirk H, Vrieling K, Mulder PPJ, Klinkhamer PGL. 2011b. The relationship between structurally different pyrrolizidine alkaloids and western flower thrips resistance in $F_2$ hybrid of *Jacobaea vulgaris* and *Jacobaea aquatica*. *Journal of Chemical Ecology* 37:1071-1080.

Cheng D, van der Meijden E, Mulder PPJ, Vrieling K, Klinkhamer PGL. 2013. Pyrrolizidine alkaloid composition influences cinnabar moth oviposition preferences in *Jacobaea* hybrids. *Journal of Chemical Ecology* 39:430-437.

Chou MW, Fu PP. 2006. Formation of DHP-derived DNA adducts in vivo from dietary supplements and Chinese herbal plant extracts containing carcinogenic pyrrolizidine alkaloids. *National Center for Toxicological Research* 22:321-327.

Cvorovi J, Ziberna L, Fornasaro S, Tramer F, Passamonti S. 2018. Bioavailability of flavonoids: the role of cell membrane transporters. In: Polyphenols: Mechanisms of Action in Human Health and Disease. 2nd Edition. Academic Press Chapter 22, pp.295-320.

Duan H, Schuler MA. 2006. Heterologous expression and strategies for encapsulation of membrane-localized plant P450s. *Phytochemistry Reviews* 5:507-523.

Dyer LA, Dodson CD, Stireman JO, Tobler MA, Smilanich AM, Fincher RM, Letourneau DK. 2003. Synergistic effects of three *Piper* amides on generalists and specialist herbivores. *Journal of Chemical Ecology* 29:2499-2514.

Ehmke A, von Borstel K, Hartmann T. 1987. Specific uptake of the *N*-oxides of pyrrolizidine alkaloids by cells, protoplasts, and vacuoles from *Senecio* cell cultures. In: Marin BP (ed) Plant vacuoles. New York: Plenum Press pp. 301-304.

Ehmke A, von Borstel K, Hartmann T. 1988. Alkaloid *N*-oxides as transport and vacuolar storage compounds of pyrrolizidine alkaloids in *Senecio vulgaris* L. *Planta* 176:83-90.

Ehrlich PR, Raven PH. 1964. Butterflies and plants: a study of coevolution. *Evolution* 18:586-608.

Firn RD, Jones CG. 2003. Natural products - a simple model to explain chemical diversity. *Natural Product Reports* 20:382-391.

Firn RD, Jones CG. 2009. A Darwinian view of metabolism: molecular properties determine fitness. *Journal of Experimental Botany* 60:719-726.

Funayama S, Cordell GA. 2015. Alkaloids: a treasury of poisons and medicines. Academic Press pp.1-20.

Futuyma DJ, Agrawal AA. 2009. Macroevolution and the biological diversity of plants and herbivores. *Proceedings of the National Academy of Sciences of the United States of America* 106:18054-18061.

Hadacek F. 2002. Secondary metabolites as plant traits: current assessment and future perspectives. *Critical Reviews in Plant Sciences* 21:273-322.

Hamann T, Møller BL. 2007. Improved cloning and expression of cytochrome P450s and cytochrome P450 reductase in yeast. *Protein Expression And Purification* 56:121-127.

Hamberger B, Bak S. 2013. Plant P450s as versatile drivers for evolution of species-specific chemical diversity. *Philosophical Transactions of the Royal Society B* 368: 20120426.

Hartmann T, Dierich B. 1998. Chemical diversity and variation of pyrrolizidine alkaloids of the senecionine type: biological need or coincidence? *Planta* 206:443-451.

Hartmann T, Ehmke A, Eilert U, von Borstel K, Theuring C. 1989. Site of synthesis, translocation and accumulation of pyrrolizidine alkaloid *N*-oxides in *Senecio vulgaris* L. *Planta* 177:98-107.

Hartmann T, Sander H, Adolph R, and Toppel G. 1988. Metabolic links between the biosynthesis of pyrrolizidine alkaloids and polyamines in root cultures of *Senecio vulgaris*. *Planta* 175:82-90.

Hartmann T, Toppel G. 1987. Senecionine *N*-oxide, the primary product of pyrrolizidine alkaloid biosynthesis in root cultures of *Senecio vulgaris*. *Phytochemistry* 26:1639-1643.

Hartmann T, Witte L. 1995. Chemistry, biology and chemoecology of the pyrrolizidine alkaloids. In Pelletier SW (ed) Alkaloids: chemical and biological perspective. Oxford: Pergamon Vol. 9, pp.155-233.

Hartmann T. 1996. Diversity and variability of plant secondary metabolism: a mechanistic view. *Entomologia Experimentalis et Applicata* 80:177-188.

Hartmann T. 1999. Chemical ecology of pyrrolizidine alkaloids. *Planta* 207:483-495.

Hasemann CA, Kurumbail RG, Boddupalli SS, Peterson JA, Deisenhofer J. 1995. Structure and function of cytochromes P450: a comparative analysis of thee crystal structures. *Structure* 3:41-62.

Havsteen BH. 2002. The biochemistry and medical significance of the flavonoids. *Pharmacology & Therapeutics* 96:67-202.

Hol WHG, Vrieling K, van Veen JA. 2003. Nutrients decrease pyrrolizidine alkaloids concentrations in *Senecio jacobaea*. *New Phytologist* 158:175-181.

Hol WHG. 2011. The effect of nutrients on pyrrolizidine alkaloids in *Senecio* plants and their interactions with herbivores and pathogens. *Phytochemisty Reviews* 10:119-126.

Isah T. 2019. Stress and defense responses in plant secondary metabolites production. *Biological research* 52:39.

Jones CG, Firn RD. 1991. On the evolution of plant secondary chemical diversity. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences* 333:273-280.

Joosten L, Cheng D, Mulder PPJ, Vrieling K, van Vee JA, Klinkhamer PGL. 2011. The genotype dependent presence of pyrrolizidine alkaloids as tertiary amine in *Jacobaea vulgaris*. *Phytochemistry* 72:214-222.

Joosten L, Mulder PPJ, Klinkhamer PGL, van Veen JA. 2009. Soil-borne microorganisms and soil-type affect pyrrolizidine alkaloids in *Jacobaea vulgaris*. *Plant and Soil* 325:133-143.

Joshi J, Vrieling K. 2005. The enemy release and EICA hypothesis revisited: incorporating the fundamental difference between specialist and generalist herbivores. *Ecology Letters* 8:704-714.

Kessler A, Halischke R. 2007. Specificity and complexity: the impact of herbivore-induced plant response on arthropod community structure. *Current Opinion in Plant Biology* 10:409-414.

Kessler A, Kalske A. 2018. Plant secondary metabolite diversity and species interactions. *Annual Review Ecology, Evolution, and Systematics* 49:115-138.

Kliebenstein DJ, Kroymann J, Brown P, Figuth A, Pedersen D, Gershenzon J, Mitchell-Olds T. 2001. Genetic control of natural variation in *Arabidopsis* glucosinolate accumulation. *Plant Physiology* 126:811-825.

Kostenko O, van de Voorde TFJ, Mulder PPJ, van der Putten, Bezemer TM. 2012. Legacy effects of aboveground-belowground interactions. *Ecology Letters* 15:813-821.

Langel D, Ober D, Pelser PB. 2011. The evolution of pyrrolizidine alkaloid biosynthesis and diversity in the Senecioneae. *Phytochemistry Reviews* 10:3-74.

Leckie BM, D'Ambrosio DA, Chappell TM, Halitschke R, De Jong DM, Kessler A, Kennedy GG, Mutschler MA. 2016. Differential and synergistic functionality of acylsugars in suppressing oviposition by insect herbivores. *PLOS ONE* 11:e0153345.

Li L, Chang Z, Pan Z, Fu Z, Wang X. 2008. Modes of heme binding and substrate access for cytochrome P450 CYP74A revealed by crystal structures of allene oxide synthase. *Proceedings of the National Academy of Sciences of the United States of America* 105:13883-13888.

Liu X, Vrieling K, Klinkhamer PGL. 2017. Interactions between plant metabolites affect herbivores: a study with pyrrolizidine alkaloids and chlorogenic acid. *Frontiers in Plant Science* 8:903.

Macel M, Klinkhamer PGL, Vrieling K, van der Meijden E. 2002. Diversity of pyrrolizidine alkaloids in *Senecio* species does not affect the specialist herbivore *Tyria jacobaea*. *Oecologia* 133:541-550.

Macel M, Vrieling K, Klinkhamer PGL. 2004. Variation in pyrrolizidine alkaloid patterns of *Senecio jacobaea*. *Phytochemistry* 65:865-873.

Maron JL, Agrawal AA, Schemske DW. 2019. Plant-herbivore coevolution and plant speciation. Ecology 100:e02704.

Mint Evolutionary Genomics Consortium. 2018. Phylogenomic mining of the mints reveals multiple mechanisms contributing to the evolution of chemical diversity in Lamiaceae. *Molecular Plant* 11:1084-1096.

Mizutani M. 2012. Impacts of diversification of cytochrome P450 on plant metabolism. *Biological and Pharmaceutical Bulletin* 35:824-832.

Moore BD, Andrew RL, Külheim C, Foley WJ. 2014. Explaining intraspecific diversity in plant secondary metabolites in an ecological context. *New Phytologist* 201:733-750.

Mulder PPJ, López P, Castelari M, Bodi D, Ronczka S, Preiss-Weigert A, These A. 2018. Occurrence of pyrrolizidine alkaloids in animal-and plant-derived food: results of a survey across Europe. *Food Additives & Contaminants: Part A* 35:118-133.

Nelson D, Werch-Reichhart D. 2011. A P450-centric view of plant evolution. *The Plant Journal* 66:194-211.

Nelson D. 2009. The cytochrome p450 homepage. *Humman Genomics* 4:59-65.

Nelson DR. 2004. Cytochrome P450 Nomenclature, 2004. In Phillips IR, Shephard EA (ed) Methods in molecular biology: cytochrome P450 protocols methods in molecular biology. 2nd edition. Totowa: Humana Press Inc. Vol 320.

Nützmann HW, Huang A, Osbourn A. 2016. Plant metabolic clusters - from genetics to genomics. *New Phytologist* 211:771-789.

Ober D, Hartmann T. 1999. Homospermidine synthase, the first pathway-specific enzyme of pyrrolizidine alkaloid biosynthesis, evolved from deoxyhypusine synthase. *Proceedings of the National Academy of Sciences of the United States of America* 96:14777-14782.

Pelser PB, de Vos H, Theuring C, Beuerle T, Vrieling K, Hartmann T. 2005. Frequent gain and loss of pyrrolizidine alkaloids in the evolution of *Senecio* section *Jacobaea* (Asteraceae). *Phytochemistry* 66:1285-1295.

Pelser PB, van den Hof K, Gravendeel B, van der Meijden R. 2004. The systematic value of morphological characters in *Senecio* Sect. *Jacobaea* (Asteraceae) as compared to DNA sequences. *Systematic Botany* 29:790-805.

Pompon D, Louerat B, Bronine A, Urban P. 1996. Yeast expression of animal and plant P450s in optimized redox environments. *Methods in Enzymology* 272:51-64.

Rasmann S, Agrawal AA. 2009. Plant defense against herbivory: progress in identifying synergism redundancy, and antagonism between resistance traits. *Current Opinion in Plant Biology* 12:473-478.

Renault H, Bassard JE, Hamberger B, Werck-Reichhart. 2014. Cytochrome P450-mediated metabolic engineering: current progress and future challenges. *Current Opinion in Plant Biology* 19:27-34.

Richards LA, Dyer LA, Forister ML, Smilanich AM, Dodson CD, Leonard MD, Jeffrey CS. 2015. Phytochemical diversity drives plant-insect community diversity. *Proceedings of the National Academy of Sciences of the United States of America* 112:10973-10978.

Sander H, Hartmann T. 1989. Site of synthesis, metabolism and translocation of senecionine *N*-oxide in cultured roots of *Senecio erucifolius*. *Plant Cell, Tissue and Organ Culture* 18:19-31.

Schuler MA, Werch-Reichhart D. 2003. Functional genomics of P450s. *Annual Review of Plant Biology* 54:629-667.

Sezutsu H, Le Goff G, Feyereisen R. 2013. Origins of P450 diversity. *Philosophical Transactions of the Royal Society B* 368:20120428.

Soldaat LL, Boutin JP, Derridj S. 1996. Species-specific composition of free amino acids on the leaf surface of four *Senecio* species. *Journal of Chemical Ecology* 22:1-12.

Toppel G, Witte L, Riebesehl B, von Borstel K, Hartmann T. 1987. Alkaloid patterns and biosynthetic capacity of root cultures from some pyrrolizidine alkaloid producing *Senecio* species. *Plant Cell Reports* 6:466-469.

Urban P, Cullin C, Pompon D. 1990. Maximizing the expression of mammalian cytochrome P-450 monooxygenase activities in yeast cells. *Biochimie* 72:463-472.

Vrieling K, de Boer N. 1999. Host-plant choice and larval growth in the cinnabar moth: do pyrrolizidine alkaloids play a role? *Entomologia Experimentalis et Applicata* 91:251-257.

Vrieling K, de Vos H, van Wijk CAM. 1993. Genetic analysis of the concentration of pyrrolizidine alkaloids in *Senecio jacobaea*. *Phytochemistry* 32:1141-1144.

Vrieling K, van Wijk CAM. 1994. Cost assessment of the production of pyrrolizidine alkaloids in ragwort (*Senecio jacobaea* L.). *Oecologia* 97:541-546.

Werck-Reichhart D, Feyereisen R. 2000. Cytochrome P450: a success story. *Genome Biology* 1:reviews3003.

Wink M. 2003. Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. *Phytochemistry* 64:3-19.

Witte L, Ehmke H, Hartmann T. 1990. Interspecific flow of pyrrolizidine alkaloids: from plants via aphids to ladybirds. *Naturwissenschaften* 77:540-543.

Witte L, Ernst L, Adam H, Hartmann T. 1992. Chemotypes of two pyrrolizidine alkaloid-containing *Senecio* species. *Phytochemistry* 31:559-565.

Ziegler J, Facchini PJ. 2008. Alkaloid biosynthesis: metabolism and trafficking. *Annual Review of Plant Biology* 59:735-769.

Ober D. 2010. Gene duplications and the time thereafter - examples from plant secondary metabolism. *Plant Biology* 12:570-577