Pyrrolizidine alkaloid variation in Jacobaea hybrids : influence on resistance against generalist and specialist insect herbivores

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Summary and conclusions

Plants produce a vast array of secondary metabolites (SMs) such as glucosides, saponins, tannins, alkaloids, essential oils and organic acids (Fraenkel, 1959). SMs are not directly involved in the growth, development, or reproduction of plants but nevertheless play an important role in plant survival because they are involved in the interactions between plants and their environment (Hartmann, 2007). The number of SMs which have been identified exceeds 100,000 (Wink, 2009). With many more SMs yet to be discovered, estimates of the total number of SMs in plants exceed 500,000 (Hadacek, 2002). The great diversity of SMs in plants is partly attributed to the numerous structurally related SMs within each major group. For instance, terpenoids are the largest group of SMs with at least 15,000-20,000 different compounds (Langenheim, 1994). More than 120 different glucosinolates have been detected in plant species of the *Capparales* and in the genus *Drypetes* (Euphorbiales) (Fahey et al, 2001). Another example is the presence of the more than 170 SMs that have been detected in *Arabidopsis thaliana*. They belong to 7 different classes of SMs and within each class there is always a large number of compounds (>10) present (D'Auria and Gershenzon, 2005). Besides a diversity of chemical structures, SMs also show high inter- and intra-species variation (Hartmann, 1996; Hartmann and Dierich, 1998; Pelser et al, 2005). SM diversity is so intriguing, that it has generated a number of hypotheses that try to explain this variety, and which are not necessarily exclusive to one another (see Hadacek, 2002; Hadacek et al, 2011 and the references there in).

Several of these hypotheses were put forward to explain the diversity of structurally related SMs within the framework of plant defense against herbivores. 1) SMs could be Selectively Neutral: Firn and Jones (2003) developed the "Screening Hypothesis" which assumes that most SMs have no function for plants and do not bring costs or benefits to the plant fitness. Nevertheless, SM diversity is maintained because it confers the likelihood of producing new active compounds. 2) SM diversity is a result of the "Arms Race" between plants and the herbivores. Newly evolved SMs may have stronger deterring or toxic effects on insect that over time have adapted to the older ones. In turn these insects may evolve mechanisms to adapt to the new SMs. This continuous cycle has resulted in the wide diversity of SMs that can be found in plants (Ehrlich and Raven, 1964). According to this theory, structurally related SMs can differ in their effects on insect herbivores and the SMs that have most recently evolved should be more effective than the older ones (Berenbaum and Feeny, 1981; Miller and Feeny, 1983). However, this trend might not be seen in specialist herbivores which can more quickly adapt to novel, more toxic analogs in their specific host plants (Cornell and Hawkins, 2003). 3) Plants benefit from the SM diversity because of the Synergistic Effects among the SMs. SMs can act synergistically towards herbivores, which means that mixtures of SMs have more toxic and/or deterrent effects on herbivores than individual SMs (Berenbaum et al, 1991; Dyer et al, 2003; Macel et al, 2005). 4) The SM diversity may be a response to the Selection from Multiple Herbivores. Structurally

related SMs may differentially affect herbivores and thus a mixture of SMs for the plant provides a better defense against a number of herbivores (Mithen et al, 1995; Juenger and Bergelson, 1998; Juenger and Bergelson, 2000; Macel et al, 2005).

Pyrrolizidine alkaloids (PAs) represent a class of typical SMs, which are constitutively formed in the plants containing them and mediating plant-herbivore interactions (Hartmann, 1999). More than 400 PAs have been identified from approx. 6000 angiosperm species (Chou and Fu, 2006). The four hypotheses mentioned above have been assessed whether they can explain the PA diversity. Previous studies using *in vitro* experiments with purified compounds have shown that the effects on herbivorous insects can differ among structurally different PAs and PAs were acting synergistically on *Spodoptera exigua* (small mottled willow moth, Macel et al, 2005). But the *in vitro* experiments were usually conducted with only a few isolated PAs, and not necessarily included the most relevant ones, because most PAs cannot be obtained commercially as pure compounds unless at a very high cost. This problem can be resolved by the use of *in vivo* experiments combined with sensitive analytical methods to detect and quantify PAs. These *in vivo* experiments can test the effects of all individual PAs in a plant simultaneously and investigate the possibility of synergy between them.

In this thesis, the PAs in *Jacobaea* (syn. *Senecio*) species were chosen as a model system to study the selective forces from insect herbivores on PA evolution. I did *in vivo* experiments with a *Jacobaea* hybrid family instead of randomly chosen genotypes from natural populations, because segregating hybrids can show large and independent variations in SM expression and herbivore resistance. Therefore, hybrids are regarded as useful tools for studying the relationship between these traits (Hochwender et al, 2000; Orians, 2000; Lexer et al, 2003). The chosen hybrid family originated from an artificial cross between *Jacobaea aquatica* (syn. *Senecio aquaticus*) and *Jacobaea vulgaris* (syn. *Senecio jacobaea*). It contains ca.100 F_2 hybrid genotypes, beside the *J. aquatica*, *J. vulgaris* and the two F_1 hybrid genotypes. The experimental chapters of this thesis consists of two parts: In the first part (Chapter 2-3) the focus was on PA variation in the *Jacobaea* hybrids and in the second part (Chapter 4-6) the resistance to insect herbivores and the influence of PAs on the herbivore resistance was studied.

1. PA variation in *Jacobaea* hybrid

The PA composition and concentration in the hybrid family was investigated in Chapter 2. The 37 individual PAs identified from the hybrid plants could be classified into four structural groups: senecionine-, jacobine-, erucifoline- and otosenine-like PAs. In the hybrids a greater PA variation was observed compared to the parents: some F_2 hybrids produced novel PA compositions and showed transgressive PA expression. For instance, floridanine was not detected in the roots of parental genotypes, but it was present in the roots of more than 60% of the F_2 hybrid genotypes. And in the F_2 hybrid shoots significant under- or over-expression of individual PAs occurred in 7.5% of all cases and in 7.5% of the cases this also occurred for the PA group or total concentrations. It was also found that within each of the four structural groups the PAs covaried with respect to concentration, but between the different structural groups the PAs showed independent segregation. In the hybrid family the PA expression displayed transgressive and independent segregation patterns as was expected.

For a long time it was assumed that PAs were present predominantly as *N*-oxides in *Senecio* (or *Jacobaea*) species and that tertiary amines were only spontaneously produced during extraction and sample clean-up (Hartmann and Toppel, 1987; Hartmann et al, 2004). In Chapter 3, it was shown that the tertiary PAs detected in the sample extracts of *J. vulgaris*, *J. aquatica* and their hybrids were not artifacts caused by the extraction procedure. It was shown that in the plants jacobine-like PAs are present in a higher proportion of tertiary amines than the other kinds of PAs. Jaconine, for instance, was present for more than 50% in the tertiary amine form, while senecionine- and erucifoline-like PAs occurred predominately (> 80%) as *N*-oxides. Moreover, in individual plants the proportion of tertiary amines was dependent on the plant genotype. The influence of genetic variation on the proportion of tertiary amines indicates that in ecological and evolutionary studies on PAs (especially of jacobine-like PAs in *Jacobaea* and other species) it may be important to discriminate between the two PAs forms.

2. the influence of PA variation on herbivore resistance

The oviposition preference of *Tyria jacobaeae* (cinnabar moth) among 40 F_2 hybrids of *J. vulgaris* and *J. aquatica* was studied in Chapter 4. *Tyria jacobaeae* is a specialist herbivore only feeding on a restricted number of *Senecio* / *Jacobaea* species. *Tyria jacobaeae* oviposited on plants from all genotypes and no PA-deterring effects on the oviposition by *T. jacobaeae* were observed. This clearly indicated that *T. jacobaeae* is well adapted to the available suit of PAs. However, it was noticed that hybrids with lower concentrations of tertiary jacobine-like PAs received fewer eggs. Moreover, for the combination of jacobine- and otosenine-like PAs, synergistic effects were found on the oviposition preference. The relationship between PA composition and concentration in the *Jacobaea* hybrids and the feeding damage from *Frankliniella occidentalis* (western flower thrips, a generalist insect herbivore) was investigated in Chapter 5. Feeding damage decreased with increasing jacobine-like (tertiary amine as well as *N*-oxide forms) PA concentration in the plants. The other structural groups did not exert any significant effect and between the PA groups no synergistic effects were found with respect to thrips resistance. The results of a bioassay with *Liriomyza trifolii* (American serpentine leafminer, a generalist insect herbivore) and plants of the hybrid family are presented in Chapter 6. A significant positive correlation was found between the plant size and the number of leafminer pupae, while correlations between PA variation and the number of pupae were rather low. Only the number of pupae per plant (corrected for plant size) decreased with increasing concentration of otosenine-like PAs. There were some indications for synergistic effects between the PAs with respect to leafminer resistance, but these effects were small.

3. Conclusion

The *Jacobaea* hybrid family turned out to be a good tool to study the relationship between PA variation and herbivore resistance because the hybrids showed great variation in both traits. By means of three bioassays, one with a specialist and two with generalist insect herbivores, I could show that *Jacobaea* hybrid genotypes differed in the resistance to these herbivores and that these differences were related to PA variation in the plants. Not all PAs equally contributed to the resistance against a herbivorous insect, and the effect of the PAs strongly depended on the herbivore tested. In all three bioassays several PAs (at least 10 out 37 PAs) seemed to be involved in the resistance to insect herbivores. These results do not meet the predictions that can be made based on the Selectively Neutral Theory, but they are more or less in support of the other three hypotheses. The thrips and leafminer bioassays both showed that evolutionary younger PAs (jacobine- and otosenine-like PAs) exerted negative effects on these herbivores, while the evolutionary older PAs (senecionine-like PAs) did not. This piece of evidence supports the Arms Race Theory. In contrast, in the cinnabar moth bioassay, no individual PA or PA combination was negatively correlated with the oviposition preference. This is an indication that generalist and specialist herbivores might play different roles with respect to SM evolution (Cornell and Hawkins, 2003).

The cinnabar moth experiment also showed that it is important to distinguish between the tertiary and *N*-oxide forms of the PAs. When we compare the three bioassays with different insects, we may conclude that the particular kind of PAs had different effects on these insects. For instance, the tertiary amines of jacobine-like PAs were positively related to the cinnabar moth oviposition preference but they were negatively related to thrips feeding. These observations are supportive for the Generalist-Specialist Dilemma, which states that qualitative defense compounds in plants will deter generalist but attract specialist herbivores and that generalist and specialist herbivores exert opposite forces on the SM concentrations (van der Meijden, 1996). The results of the three bioassays and their relevance regarding the four hypotheses are summarized in Table 1.

Table 1 Summary of the relationships between pyrrolizidine alkaloid (PA) concentrations and the behavior of insect herbivores in three bioassays conducted with *Jacobaea* F2 hybrids. The insects selected are: Cinnabar moth (*Tyria jacobaeae*), Western flower thrips (*Frankliniella occidentalis*), and American serpentine leafminer (*Liriomyza trifolii*). Data used are the genotypic mean values. Numbers in the cells are the *r* values from Pearson correlation tests.

	Insect herbivore	Cinnabar moth (Egg batch, N=40)	Thrips (feeding damage, N=98)	Leafminer (pupae/plant size, N=90)
PA concentration	Total PA (tertiary amines)	0.47 **	-0.32 **	-0.08 ns
	Total PA (*N*-oxides)	0.13 ns	-0.28 **	-0.05 ns
	Senecionine-like PAs	-0.06 ns	-0.18 ns	-0.16 ns
	Jacobine-like PAs (tertiary amines)	0.47 **	-0.30 **	0.09 ns
	Jacobine-like PAs (*N*-oxides)	0.13 ns	-0.34 ***	0.08 ns
	Erucifoline-like PAs	-0.06 ns	-0.16 ns	0.24 *
	Otosenine-like PAs	0.19 ns	0.03 ns	-0.33 ***
Hypotheses	Selectively neutral theory	-	-	-
	Arms race theory	+	+	+
	Synergistic effects among PAs	+	-	?
	PAs' effects different among herbivores	+	+	+

Significance codes: ns; * P: 0.01-0.05; ** P: 0.001-0.01; *** P: < 0.001.
+,-: Assay provides support / provides no support for the specific hypothesis. ?: From the assay no conclusion can be drawn with regard to the specific hypothesis.

With respect to the development of new methods to study the ecology and evolution of PAs in *Jacobaea* species, this thesis can be seen as a continuation of the work carried out by others (e.g. Macel et al, 2005; Leiss et al, 2009; Kirk et al, 2010). By analysis of the relationship between PA variation and herbivore resistance in *Jacobaea* hybrids it could be shown that plants can benefit from PA diversity when the environment imposes multiple stresses, such as with multiple insect herbivores, because the effects that PAs can have on herbivore insects are differential and probably also synergistic. Meanwhile, the large PA variation, which is genetically controlled, may be helpful for plants to adapt to frequent changes in the environment.

To present a more complete picture of PA evolution, further insight can be obtained by a systems biology approach studying the: 1) the underlying genetics of PA production, e.g. the biosynthetic pathway of PAs and its regulation; 2) the physiological processes related to PA production, translocation and accumulation; 3) the interaction between PAs and other defense or resistance traits, such as other defense compounds and plant tolerance to herbivory such as re-growth; 4) the effect of PAs on multiple herbivores, pathogens and interactions through different trophic levels; 5) other biotic and abiotic environmental factors influencing PA variation.

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