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

Cheng, D.

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Influence on resistance against generalist and specialist insect herbivores

by Dandan Cheng

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Prof. Dr. E. van der Meijden
Prof. Dr. N.M. van Dam
Radboud University Nijmegen, The Netherlands
Dr. M. Macel
University of Tübingen, Germany

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Chapter 1

General introduction

Plants produce a high diversity of secondary metabolites (SMs). The number of SMs which have been identified exceeds 100 000 (Wink, 2009) and the chemical structures of at least 47 000 SMs have been described (De Luca and St Pierre, 2000). With many more SMs yet to be discovered, estimates of the total number of SMs in plants exceed 500,000 (Hadacek, 2002). Within a particular species, or individual plant, a number of major SMs are usually accompanied by several derivatives as minor components (Wink, 2003). For instance, 34 glucosinolates were found in *Arabidopsis thaliana* (Kliebenstein et al, 2001) and more than 20 indole alkaloids were produced in hairy root culture from *Rauvolfia serpintina* (Sheludko et al, 2002). Beside the structural diversity, SMs often show a large variation in concentration. A good example is the variation in the total concentration of the aliphatic glucosinolates in leaves of the ecotypes of *A. thaliana*, which varied nearly 20 fold in total concentration (Kliebenstein et al, 2001). Qualitative and quantitative variation of SMs in plants is determined by genetics (Vrieling et al, 1993; van Dam and Vrieling, 1994; Kliebenstein et al, 2001; Macel et al, 2004), the environment and the interaction between these two (Arany et al, 2009; Lankau and Kliebenstein, 2009; Kirk et al, 2010). As yet, it is poorly understood from an evolutionary point of view how SM diversity emerged and why it is maintained in nature.

In this thesis I will study this evolutionary question from a perspective of the SMs' functions. As a study system I have chosen the pyrrolizidine alkaloids (PAs) of *Jacobaea* (syn. *Senecio*) species. I will investigate whether structurally related PAs in *Jacobaea* species can differentially influence the plants resistance against specialist and generalist insect herbivores. I will study the variation in PA composition and concentration among circa 100 F_2 hybrid genotypes of a cross between *Jacobaea aquatica* (syn. *Senecio aquaticus*) and *Jacobaea vulgaris* (syn. *Senecio jacobaea*). Making use of the independent segregation of different structural types of PAs, I will study the effect of PAs on plant resistance against generalist and specialist insect herbivores.

1. Secondary metabolites

In 1873, Julius Sachs, one of the founders of plant physiology realized that plants contained substances with no obvious function. In 1891, plant physiologist Albrecht Kossel designated the term "secondary" for these low-molecular weight and seemingly non-functional metabolites present in plants. Almost in the same period, others, such as Anton Kerner von Marilaun, Ernst Stahl and Leo Errera found that secondary metabolites protected plants from attack of animals (see reviews by Hadacek, 2002; Hartmann, 2007 and 2008).

Classes of compounds that are regarded as SMs include, amongst others, glucosides, saponins, tannins, alkaloids, essential oils and organic acids. These compounds differ from primary chemicals with respect to function and occurrence. SMs are not directly involved in the growth, development, or reproduction of the plant. Very often they occur in specific taxons (Fraenkel, 1959). But the distinction between primary and secondary metabolism is blurred. Firstly, not all primary metabolites (PMs)

occur in every plant, although primary metabolism involves the essential reactions that occur in all different groups of living organisms. Secondly, some SMs may also function as co-substrates or co-enzymes in primary metabolism (Hadacek, 2002).

Hartmann (1996 and 2007) suggested to base the definition of primary and secondary metabolites on their functions. Thus PMs are universal, conservative, and indispensable chemicals, while SMs are exclusive, diverse, and while dispensable for growth and development, they are indispensable for survival. According to this definition, a metabolite may be a PM as well as a SM. For instance, canavanine from jack bean (*Canavalia ensiformis*) has this dual role as a PM and a SM, because it is both an efficient defense and a nitrogen storage compound (Rosenthal and Rosenthal, 1982).

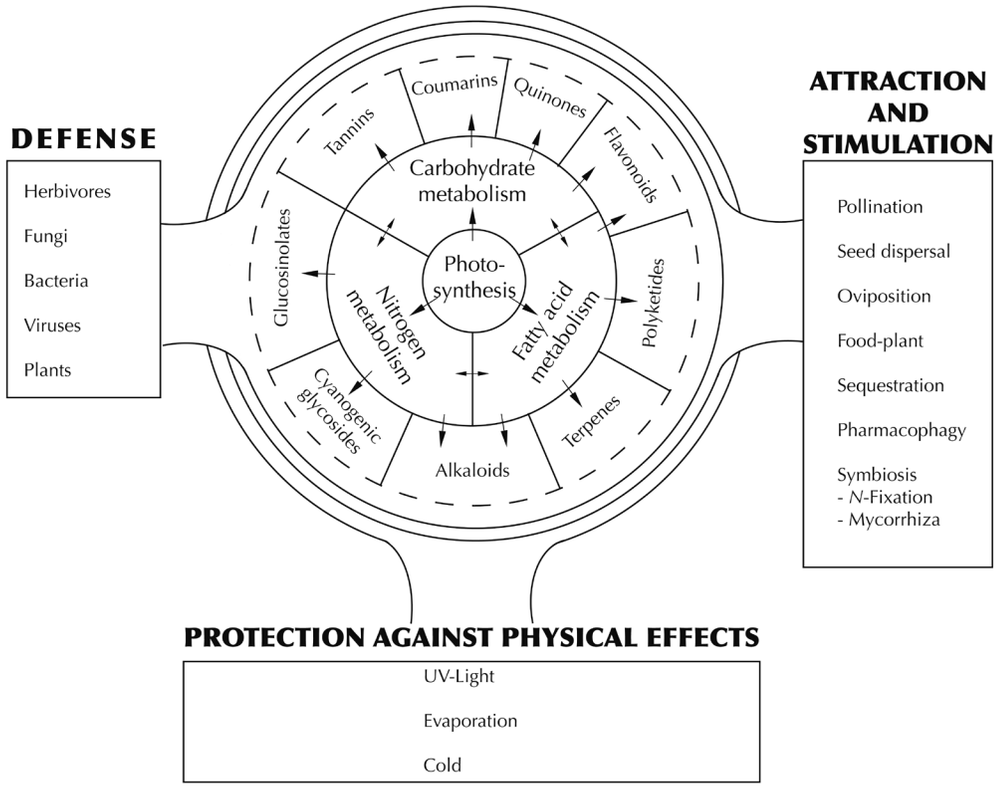

Fig.1 Ecological functions of plant secondary metabolism (from Hartmann, 1996)

1.1. Function of secondary metabolites (SMs) in plants

The idea that SMs have important functions for plants has been widely accepted since the 1970s (Harborne, 1972; Swain, 1977). The functional aspects of plant SMs are illustrated in Fig.1 as proposed by Hartmann (1996). This scheme also illustrates the major groups of SMs and that SMs originate from common precursors of primary metabolism. SMs are involved in all the interactions between plants and their environment (biotic and abiotic). SMs may act as defense compounds against antagonistic organisms such as microbiological attackers, invertebrate and vertebrate herbivores and competing plants. In contrast, specialist herbivores adapted to plant defense can use particular SMs as cue for locating food-plants, oviposition and even utilize SMs for their own benefit. SMs are signaling chemicals in communication with potentially beneficial (and non-beneficial) organisms. They may attract pollinators, seed dispersers (see Hadacek, 2002 and references therein). In addition, SMs may act as protection against abiotic stresses such as high levels of UV radiation, temperature and drought stress (Chen et al, 2009; Vickers et al, 2009). It was also found that plants can release distinct volatile bouquets of SMs when attacked and these SMs can function as indirect defense compounds by attracting carnivores as was shown in controlled experiments in the greenhouse (reviewed by Allison and Hare, 2009) and in the field (Allmann and Baldwin, 2010).

1.2. Hypotheses to explain secondary metabolites (SM) diversity

Several theories and hypotheses have been put forward to explain the SM diversity in plants from the perspective of the function of SMs in plant defense against herbivores. Although these hypotheses are dealing with the diversification of all classes of SMs, they can be used to explain the diversity in structurally related SMs of a specific class. After all, the diversity within a major group of SMs is generally greater than across different kinds of SMs (Langenheim, 1994). For instance, in *A. thaliana* there are more than 170 SMs belonging to 7 different classes, each class containing more than 10 different compounds (D'Auria and Gershenzon, 2005).

Firstly, the SM diversity could be the result of Neutral Selectivity. Firn and Jones (2003 and 2009, see also Jones and Firn, 1991) developed the "Screening Hypothesis" to explain the evolution of plant SM diversity. Unlike the theories which emphasize the SMs' function against herbivores, their hypothesis states that most SMs (perhaps more than 90%, as has been estimated from commercial screening programs) have no distinct function for the plants and provide neither cost nor benefit in relation to plant fitness. In other words, most individual SMs are of neutral selection. Nevertheless, SM diversity is favored because it confers the likelihood of producing new active compounds. This can be compared to some extent to commercial screening programs that search for novel bio-active compounds to certain receptor targets. In the screening programs the chance of success is directly related to the number of chemicals that can be screened in a short time, due to a very low frequency of a good match. It is not easy to test this hypothesis in a direct way, because it is difficult to exactly identify the function of each particular SM in plants. However, by applying this hypothesis for a group of SMs, we can test how many compounds from this particular group have an effect on herbivores and how many do not. If a high proportion of SMs (say more than 10%) shows a repellent effect, then the theory of neutral selectivity may not be true for this group of SMs. In contrast to the Screening Hypothesis, many SMs, as for instance, PAs in *Senecio* species, are generally regarded as powerful defense compounds in plants (e.g. Hartmann, 1996).

Secondly, it was hypothesized that the SM diversity was caused by the "Arms Race" between plants and the herbivores. Ehrlich and Raven (1964) proposed that novel SMs increase plant fitness because of the reduction of herbivory. Plant species with the novel SMs have entered a new adaptive zone and the evolutionary radiation might follow. In turn, herbivore species that succeed to counter adapt to these SMs, gain greater fitness and evolutionary radiation happened. The next step is that plants again evolve new SMs. The diversity of plants, herbivorous insects and the SMs that we observe today could be the result of this sequence of evolutionary events. Applying this theory to explain the diversity within a structurally related group of SMs, it follows that individual SMs should differ in their effects on insect herbivores and the SMs that have most recently evolved are more effective than the

older ones. This theory is supported by some experimental evidence (e.g. Berenbaum and Feeny, 1981; Miller and Feeny, 1983, Macel et al, 2005). Cornell and Hawkins (2003) stated that herbivorous insects can adapt to the SMs in host plants, so it may be expected that the more widespread SMs are less toxic than the more narrowly distributed ones. This trend will be stronger with generalist than with specialist herbivores, due to the different adaption strategies of these two groups. Specialist herbivores can quickly adapt to novel, more toxic metabolites in specific host plants, while generalists can tolerate less toxic chemicals by means of feeding on many different hosts and in this way reducing their toxic burden. The Arms Race Theory and other hypotheses of phytochemical coevolution have been criticized on a number of points and some researchers doubt whether insect herbivores could be a selective force on plant SMs (e.g.Thompson, 1988; Jermy, 1993). However, in general, the concept of coevolution has been enthusiastically adopted and it has evoked a lot of new ideas for further development or modification (Futuyma and Agrawal, 2009; Janz, 2011)

Thirdly, the SM diversity could be explained by the acting of Synergistic Effects among SMs. If different compounds can act synergistically on herbivores, then a mixture of structurally related SMs could have a more toxic and deterrent effect on herbivores than the SMs individually. Therefore, plants obtain a benefit if they maintain a high diversity of SMs (Berenbaum et al, 1991; Dyer et al, 2003; Macel et al, 2005).

Finally, the SM diversity may be a result of the Selection from Multiple Herbivores. It can be assumed that each specific SM provides resistance to one or a number of specific herbivores. Several studies have revealed that the relative effects of related compounds may differently affect generalist insect herbivores (Mithen et al, 1995; Juenger and Bergelson, 1998; Juenger and Bergelson, 2000; Macel et al, 2005; Iason et al, 2011). Hence, a mixture of SMs could be selectively advantages by protecting the plants under the pressure from multiple herbivore species including specialists and generalists.

Beside the variation of SM compositions in plants there is a high variation in concentration of SMs, which could be explained by the balance between benefits and direct costs or ecological costs of SM production. Plants benefit from the SMs because the SMs protect them from herbivores, leading to an increased fitness. On the other hand, production of SMs may reduce the plants' investment to growth and reproduction. This is called The Direct Cost of Resistance. The trade-off between costs and benefits of SM production can be used to explain the variation of SMs maintained in plants (Coley et al, 1985; Herms and Mattson, 1992). Several studies provide support for this idea; while others did not find fitness costs of plant defense (see reviews by Bergelson and Purrington, 1996; Koricheva, 2002; Strauss et al, 2002). In the case of PAs in *Jacobaea* species, costs related to the PA production were regarded absent or small (Vrieling and and van Wijk, 1994a; Vrieling and van Wijk, 1994b; Vrieling et al, 1996). In some cases, the costs of the production of SMs may be not direct, but rather indirect, e.g. may result from increased damage by specialist herbivores. SMs defend plants from generalist herbivores but at the same time they may attract specialist herbivores. The variation in SM concentration in plants can thus be explained by the opposing effect of specialist and generalist herbivores, which is called the Generalist-Specialist Dilemma (van der Meijden, 1996). This hypothesis is supported by the experimental evidence that in plants the concentration of defense chemicals shifted according to the pressure from specialist and generalist herbivores (Lankau, 2007; Arany et al, 2008). Finally, costs could also arise from the negative effects that SMs can exert on beneficial organisms (van der Meijden, 1996). However, this aspect will not be a topic of this thesis.

PAs produced in a hybrid system from a cross between two *Jacobaea* species were chosen as a model to study the SM evolution. PAs from *Jacobaea* species are a well-documented group of SMs and PA biosynthesis has been intensively studied. However, the evolutionary basis of the PA diversity is not clear yet (Langel et al, 2011).

2. Pyrrolizidine alkaloids (PAs)

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), of which more than 95% belong to four families: Asteraceae, Boraginaceae, Fabaceae and Orchidaceae (Langel et al, 2011).

PAs can occur in plants in two forms: tertiary amine (free base) and *N*-oxide (Rizk, 1991; Wiedenfeld et al, 2008). Hartmann and coworkers showed that PAs are produced as *N*-oxides in the roots and are predominantly present as *N*-oxides in *Senecio* species. The (partial) reduction of *N*-oxides to corresponding tertiary amines can happen spontaneously during alkaloid extraction, resulting in an artificially high amount of tertiary PAs in the sample (Hartmann and Zimmer, 1986; Hartmann and Toppel, 1987, Hösch et al, 1996). However, more recent research indicated that not all PAs are exclusively present as *N*-oxides in the shoots of vegetative *J. vulgaris* plants. Some jacobine-like PAs (jacobine, jacoline, jaconine and jacozine) can regularly occur up to 50% as tertiary amines (Joosten et al, 2009). I will investigate the occurrence of tertiary amines in more detail in Chapter 3.

2.1 PA biosynthesis, translocation and accumulation in Jacobaea/Senecio plants

Pelser et al (2005) reported that 26 PAs (as tertiary amines) were present in 24 species of sect - *Jacobaea*. In *Jacobae*a species, all PAs except senecivernine are derived from senecionine *N*-oxide; senecionine *N*-oxide is synthesized in the roots, via the phloem transported to the shoots, where it is diversified into other PA structures (Hartmann and Toppel, 1987; Hartmann et al, 1989). Aside from structural diversification, PAs do not undergo any significant turnover or degradation (Hartmann and Dierich, 1998). The diversity from senecionine *N*-oxide to other PAs comprises simple one-step or two-step reactions such as hydroxylations, epoxidations, dehydrogenations, and O-acetylations, as well as the more complex conversion of the retronecine into the otonecine base moiety (Hartmann and Dierich, 1998). The first specific compound of PA biosynthesis was identified as homospermidine, which is converted to spermidine and putrescine by the enzyme homospermidine synthase (HSS) (Böttcher et al, 1993). It was shown that the HSS encoding gene originated by gene duplication (Ober and Hartmann, 1999), independently in unrelated angiosperm families (Reimann et al, 2004). The enzymes responsible for the PA diversification are not identified yet. It has been suggested that the genes encoding for the PA pathway-specific enzymes are regulated by a switch-off and switch-on mechanism rather than by gain and loss, since PA distribution appears to be largely incidental in *Senecio* species (Pelser et al, 2005). A schematic diagram representing putative PA biosynthetic pathways is shown in Appendix 1 and the chemical structures of PAs detected in the *Jacobaea* hybrid system that has been used in this study are shown in Appendix 2.

PA accumulation in a particular tissue depends on a number of interacting processes: (i) synthesis of senecionine *N*-oxide in roots, (ii) continuous long-distance translocation of senecionine *N*-oxide into shoots, (iii) differential senecionine *N*-oxide transformations in different plant organs, (iv) continuous allocation of PAs in the plant, and (v) tissue selective vacuolar storage of PAs (reviewed

by Hartmann and Dierich, 1998). In *Jacobaea erucifolia* (syn. *Senecio erucifolius*), a closely related species of *J. vulgaris*, PA biosynthesis occurs mainly in the root apex and thus coincides with the site of active root growth (Sander and Hartmann, 1989). This is in line with the finding that in young *J. vulgaris* plants the total PA amount in plants was positively correlated to root biomass but negatively correlated to shoot to root ratio, which suggested that PAs are produced by roots at a root-biomass dependent rate and the greater the shoot to root ratio, the greater the overall dilution of alkaloids (Hol et al, 2003;Schaffner et al, 2003).

2.2. The function of PAs in plant defense against herbivores and pathogens

2.2.1. The effect of PAs on vertebrates

PAs are toxic for most vertebrates. Already decades ago, it was understood that, upon ingestion, in particular 1,2-unsaturated PAs were toxic to livestock and humans by causing damage to organs such as liver, lungs, and blood vessels. Most severe damage often occurs to the liver (Bull et al, 1968; McLean, 1970). PAs are not toxic themselves, but require metabolic activation (Mattocks, 1968; Fu et al, 2004; Wiedenfeld, 2011). After ingestion and absorption of PAs, the cytochrome P-450 monoxogenase enzyme complex in the liver can introduce a hydroxyl-group adjacent to the nitrogen-atom in the necine ring system. These hydroxy-PAs are unstable and are rapidly dehydrated to the corresponding 3,4-dehydropyrrolizidine alkaloids (DHPAs). Ring opening at C-7 will produce a stabilized carbonium ion that can react with the nucleophiles such as mercapto, hydroxy and amino functional groups. Such functional groups are present for instance in proteins and PA-protein adducts will be formed *in vivo*. They also react with the amino groups of purine and pyrimidine bases present in DNA and RNA (Fu et al, 2004). The alkylated products can lead to abnormal functions and alkylation of DNA may produce mutations which in the end may result in genotoxic and tumorigenic effects. PA *N*-oxides can be reduced by bacteria and enzymes present in the gut or by the liver microsomes to the corresponding tertiary PAs and they show similar toxicity as the tertiary PAs. Therefore both forms of PAs are considered carcinogenic, mutagenic, genotoxic, fetotoxic and teratogenic (Mattocks, 1968; Mattocks, 1971; Mattocks, 1986; Fu et al, 2004, Wiedenfeld, 2011).

Major detoxification pathways *in vivo* of PAs are: hydrolysis of the ester bonds in PAs leading to necic acids and the necine bases which are not toxic; *N*-oxidation by cytochrome P-450 yielding PA *N*-oxides which are highly water soluble and easily excreted (Mattocks, 1986). Hence, the toxicity level of PAs will depend on: 1)the efficiency of metabolic activation to form DHPAs and the corresponding carbonium ions and: 2) the efficiency of detoxification by ester hydrolysis or *N*-oxidation and excretion via urine (Wiedenfeld and Edgar, 2011). This could explain why the effects of PAs on animals are structure-related and that the sensitivity to PAs is different among different animal species (enzymes involved in PA metabolism differ among species). For instance, typical macrocyclic diesters PAs (e.g. PAs in *J. vulgaris*) are regarded to be more toxic than monoester PAs (e.g. PAs derived from the necine supinidine) (Wiedenfeld and Edgar, 2011). The 1,2-saturated PAs (e.g. PAs derived from the platynecine) are not genotoxic to mammalians (Wiedenfeld et al, 2008).

Contamination with PAs of livestock forage, honey and pollen (Deinzer et al, 1977; Kempf et al, 2010; Dübecke et al, 2011), herbal medicine and tea (Wiedenfeld and Edgar, 2011), and even milk (Dickinson et al, 1976; Deinzer et al, 1982; Hoogenboom et al, 2011) has been reported. PA-containing plants may contaminate food or they may be consumed as vegetables by mistake (Wiedenfeld and Edgar, 2011). Therefore, much attention has been paid to PAs because of their potential threat to human and animal health (Boppré, 2011),

2.2.2. The effect of PAs on invertebrates

The toxicity mechanism of PAs to insect herbivores is not as clear as that to mammals. Frey et al (1992) suggested that the same mechanisms may be involved for both kinds of animals. Bioassays have demonstrated that structurally different PAs differentially affect insect herbivores. A particular PA that was effective against one insect did not necessarily deter other insect species (Bentley et al, 1984; Dreyer et al, 1985; van Dam et al, 1995; Macel et al, 2005; Dominguez et al, 2008). PA mixtures often have a stronger effect on insects compared to individual PAs, indicating the presence of synergistic effects (Macel et al, 2005). Generally, the PA *N*-oxides are less deterrent than the corresponding tertiary PAs (Dreyer et al, 1985; van Dam et al, 1995; Macel et al, 2005).

PA-adapted specialist insects detoxify PAs through *N*-oxidation (Lindigkeit et al, 1997; Naumann et al, 2002). PAs may stimulate the feeding and oviposition of the larva and adult of specialist insects (Boppré, 1986; Honda et al, 1997; Kelley et al, 2002; Macel and Vrieling, 2003; Bernays et al, 2004). The oviposition-stimulating effect of PAs on *T. jacobaeae* was observed to be different among structurally different PAs but concentration-dependent effects were not found (Macel and Vrieling, 2003). Larval feeding-preference of specialist insects (the sawfly *Aglaostigma discolour* and the beetle *Oreina cacaliae*) on a PA-containing plant species (*Adenostyles alliariae*) was not affected by the isolated PAs individually added to artificial diets (Hagele and Rowell-Rahier, 2000). Some specialist insects take up PAs from the host plants and utilize them for their own defense or as sexual pheromones (see review by Trigo, 2011). *Tyria jacobaeae* even produces its own specific PAs by metabolizing PAs that were taken up from host plants (Rothschild et al, 1979).

There are some *in vivo* studies which indicated the negative influence of PAs in plants on generalist herbivorous insects. Negative correlations between concentration of total PAs and of individual PAs such as jacobine and the resistance to the generalist insect herbivore *Franklinella occidentalis* were observed in *J. vulgaris* and the hybrids of *J. vulgaris* and *J. aquatica* (Macel, 2003; Leiss et al, 2009). In field experiments, the total amount of PAs in plants of *J. vulgaris* was negatively correlated to the performance of the generalist aphid *Brachycaudus cardii* and the specialist aphid *Aphis jacobaeae* (Vrieling et al, 1991). It has also been observed that the young leaves of *J. vulgaris* plants were less damaged by generalist herbivorous insects than the old leaves, and the young leaves always contained higher amounts of PAs than the older leaves (de Boer, 1999; Leiss et al, 2009).

No relationship was found between the PAs in *J. vulgaris* plants and the specialist *T. jacobaeae* with regard to both preference and performance in bioassays under controlled conditions (Vrieling and de Boer, 1999). Similar results were found in a bioassay with *T. jacobaeae* and eight *Senecio* species (Macel et al, 2002). However, in a field study Macel and Klinkhamer (2010) found that the damage that was mainly caused by specialist herbivorous insects such as *T. jacobaeae, Longitarsus jacobaeae* and *Haplothrips senecionis*, on *J. vulgaris* plants was positively correlated to the concentration of total PAs and individual PAs (jacobine and jacobine *N*-oxide). This finding suggests that plants with higher concentrations of PAs are more attractive to the specialists. The studies published thus far seem to contradict one another. It is not clear yet whether PA variation in plants has an effect on specialist herbivores preference and performance.

2.2.3. The effect of PAs on pathogens

It was demonstrated by means of *in-vitro* bioassays that PAs isolated from plants can significantly inhibit the growth of many different fungal species (Jain and Sharma, 1987; Marquina et al, 1989; Reina et al, 1995; Reina et al, 1997; Reina et al, 1998; Hol and van Veen, 2002; Singh et al, 2002). It was shown that rhizosphere fungal communities were influenced by the PA content and composition of *J. vulgaris*. High PA concentrations decreased the diversity in the rhizosphere (Kowalchuk et al, 2006). At the other hand, there are indications that soil-born microorganisms can influence the concentration of individual PAs in *J. vulgaris* (Joosten et al, 2009).

2.2.4. PAs and plant inducible defense against aboveground herbivores

It has been suggested that whether or not PAs are involved in inducible defense depends on the life history and ecological environment of the plants (van Dam et al, 1993). In general, the difference in constitutive PA levels in *J. vulgaris* was greater than that caused by induction. It was observed that the total PA concentration in *J. vulgaris* decreased within 6-12 h, but returned to the initial value 24 h after the mechanically induced damage (van Dam et al, 1993). Vrieling and Bruin (1987) did not found a significant change of total PA concentrations in the shoots of *J. vulgaris* after artificial damage. Aboveground herbivory did not change the total concentration of PAs in the shoots, and the effect on PA composition was genotype-dependent (Hol et al, 2004).

2. 3. PA variation in *Jacobaea/Senecio* plants

2.3.1. Inter-species variation

A large variety of PA profiles can be found among *Senecio* and *Jacobaea* species (Langel et al, 2011). PA profiles are species-specific (Hartmann and Dierich, 1998). For instance, some species are very rich in jacobine-like PAs such as *J. vulgaris*, while erucifoline-like PAs dominate in *J. erucifolia*. However, PA profiles do not represent the phylogenetic relationships between the *Senecio/Jacobaea* species. This indicates that the flexible PA profiles in these species are probably helpful to protect plants from multiple herbivores, because flexible PA mixtures are more difficultly adapted by herbivores (Pelser et al, 2005; Langel et al, 2011).

2.3.2. Intra-species variation

PA profiles also vary within species. The existence of different chemotypes of *J. vulgaris* is a well known example for the intra-species PA variation. Based on the evaluation of the PA profiles of more than 100 *J. vulgaris* populations in Europe, it was concluded that there existed two different chemotypes: the 'jacobine chemotype', which is dominated by jacobine and its derivatives as major PAs; the 'erucifoline chemotype', dominated by erucifoline-like PAs (Witte et al, 1992). Besides these two chemotypes, later on also a 'senecionine chemotype' (with senecionine-like PAs as dominating PAs) and a 'mixed chemotype' (with both jacobine- and erucifoline-like PAs as dominating PAs) were described (Macel et al, 2004). The distribution of the chemotypes showed a geographic pattern: The jacobine chemotype mostly occurs in the coastal areas and the erucifoline chemotype is mainly found inland of Europe (Witte et al, 1992; Vrieling and de Boer, 1999; Macel et al, 2004). Plants from the same population often belong to the same chemotype but still they shown significant variation in relation to PA composition. For instance, although plants collected at Meijendel (the Netherlands) mainly contained jacobine, the percentage of jacobine ranged from 41 to 100% of the total PA content and the percentage of erucifoline ranged from 0 to 19% (Macel et al, 2004).

2.3.3. Intra-plant variation

PAs are not equally distributed over the organs of individual plants. PAs are stored in vacuoles and typically accumulate in the inflorescences and the peripheral stem tissues, i.e. epidermal and sub-epidermal cell layers in the plants, as has been shown for *Senecio vulgaris* (Hartmann et al, 1989). The total concentration of PAs in vegetative *J. vulgaris* plants was found to decrease with leaf age (de Boer, 1999), and inflorescences often have a higher concentration of PAs than the leaves in reproductive *J. vulgaris* plants (Witte et al, 1992).

PA composition differs in the root and shoot of the vegetative plants of *J. vulgaris*, *J. aquatica* and the F_2 hybrids: Generally, shoots have more variation in the composition and more jacobine-like PAs compared to the roots (Joosten et al, 2009). In reproductive *J. vulgaris* plants, leaves have less senecionine-like PAs but more jacobine- or erucifoline-like PAs. In *J. vulgaris* erucifoline chemotype the proportion of acetylerucifoline was however much higher in the leaves than in inflorescences (Witte et al, 1992).

The PA concentration on the leaf surface of *J. vulgaris* is much lower (less than 1%) compared to the interior of the leaves. The concentration at the surface of the leaves was only marginally correlated with that of the interior, and the PA composition on the leaf surface also differed from the PA spectrum inside (Vrieling and Derridj, 2003).

2.3.4. Genetic control and environmental influence on PA variation

It has been estimated that under climate room conditions 50-100% of the variation in total PA concentration is due to genetic variation (Vrieling et al, 1993). PA measurements on replicated genotypes illustrated that the PA concentration and composition were genotype-dependent (Macel et al, 2004; Joosten et al, 2009). PA accumulation in plants is also affected by abiotic environmental factors such as nutrients and water. It was found that *J. vulgaris* plants grown under drought or nutrient stress conditions tend to have higher PA concentrations, than those grown under normal conditions (Vrieling and van Wijk, 1994). Increased nutrient availability leads to a significant reduction in total PA concentration in shoots of *J. vulgaris* plants (Hol et al, 2003). It was postulated that in this particular situation of rich nutrient supply, the decreased PA level in shoots may have resulted from a dilution effect: The increase of the nutrient supply will favor an increase of shoot over root biomass ratio and as PA production is correlated with root growth, plants under nutrient rich conditions produce relatively less PAs. Some genotypes of *J. vulgaris*, *J. aquatica* and their hybrids produce different PA concentrations and compositions under different nutrient and water treatment conditions, so it seems that PA expression is affected by genotype and environment interactions (Kirk et al, 2010).

3. Research questions

In this thesis, I will investigate whether the structurally related PAs differentially influenced generalist and specialist insect herbivores in *Jacobae*a hybrids. I will address the following questions:

1. Do the F_2 hybrids from a cross between *J. vulgaris* and *J. aquatica* display a greater PA variation compared to their parents? Is PA variation, especially the production of tertiary PAs, dependent

on the plant genotype?
2. Is herbivore resistance against generalist and specialist insect herbivores dependent on the plant hybrid genotype?
3. Does herbivores resistance against generalist and specialist insect herbivores relate to PA composition and concentration?
4. Do the effects of PAs on herbivore resistance differ among different PAs and does it make a difference whether they are present as tertiary amines or *N*-oxides?
5. Do different PAs act synergistically in their effects on herbivores?

Up to now, most conclusions about the effects that PAs have on insects and pathogens are based on *in vitro* experiments, while these effects of PAs are not always apparent in *in vivo* experiments (see reviews by Joosten and van Veen, 2011; Macel, 2011; Trigo, 2011). Also, most of the previous studies were hampered by the fact that less sensitive methods were used to detect PAs and often no distinction was made between tertiary amines and *N*-oxides. Research on individual PAs is difficult as the majority of the PAs cannot be obtained commercially unless at a very high cost. *In vivo* experiments have an advantage over *in vitro* bioassays, in the sense that these can overcome the need for PAs as isolated compounds. The disadvantage of *in vivo* experiments is that the species or genotypes that are used may differ in other characteristics as well that are relevant for herbivory. Therefore it can be difficult to sort out the effect of PAs. Many of these disadvantages can be overcome by using segregating hybrids instead of randomly chosen genotypes: Firstly, a greater variation of SMs and herbivore resistance can occur among these hybrids compared to genotypes within a single species (Fritz, 1999; Orians, 2000; Cheng et al, 2011). Secondly, traits will segregate independently so that trait variation can be studied against an on average equal genetic background (Hochwender et al, 2000; Lexer et al, 2003). Therefore, I will use *Jacobaea* hybrids as a study system.

4. Outline of the thesis

In Chapter 2, the PA variation in the shoots and roots of F_2 hybrids, obtained from a cross between *J. vulgaris* and *J. aquatica*, will be studied. I will investigate whether there are any novel PAs, or novel PA compositions, present in the hybrids, and whether transgressive segregation of PA concentrations occurs. I will investigate whether the PA expression is different among the plant genotypes. The PA variation patterns and the implications for PA biosynthesis and PA genetic control will be discussed. For a long time it has been assumed that in *Senecio* species PAs are present mainly as *N*-oxides and that tertiary amines were mostly artifacts formed by (spontaneous) reduction of *N*-oxides during PA extraction. The presence of significant amounts of specific tertiary PAs in the plants of *J. vulgaris*, *J. aquatica* and their hybrids will be described and discussed in Chapter 3.

The oviposition preference of *T. jacobaeae* among the hybrids of *J. vulgaris* and *J. aquatica* is studied in Chapter 4. The resistance of *Jacobaea* hybrids against two generalist insect herbivores, *F. occidentalis* (western flower thrips) and *Liriomyza trifolii* (American serpentine leafminer), will be studied in Chapter 5 and Chapter 6, respectively. Through the use of bioassays, I will explore the relationship between herbivore resistance and PA variation and the possible synergism among PAs with respect to plant resistance against these insects.

Finally, the relation between PA variation in the *Jacobaea* hybrids and performance and preference of insects among the plants will be discussed and the conclusions will be summarized in the last chapter (Chapter 7).

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Chapter 2

Pyrrolizidine alkaloid variation in shoots and roots of segregating hybrids between *Jacobaea vulgaris* and *Jacobaea aquatica*

Dandan Cheng, Heather Kirk, Patrick P.J. Mulder, Klaas Vrieling, Peter G.L. Klinkhamer

Hybridization can lead to novel qualitative or quantitative variation of secondary metabolite (SM) expression that can have ecological and evolutionary consequences.

We measured pyrrolizidine alkaloid (PA) expression in the shoots and roots of a family including one *Jacobaea vulgaris* genotype and one *J. aquatica* genotype (parental genotypes), two F_1 hybrid genotypes, and 102 F_2 hybrid genotypes using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

We detected 37 PAs in the roots and shoots of *J. vulgaris, J. aquatica* and hybrids. PA concentrations and compositions differed between genotypes, and between roots and shoots. Three otosenine-like PAs that only occurred in the shoots of parental genotypes were present in the roots of F_2 hybrids; PA compositions were sometimes novel in F_2 hybrids compared to parental genotypes, and in some cases transgressive PA expression occurred. We also found that PAs from within structural groups covaried both in the roots and shoots, and that PA expression was correlated between shoots and roots.

Considerable and novel variation present among F_2 hybrids indicate that hybridization has a potential role in the evolution of PA diversity in the genus *Jacobaea*, and this hybrid system is useful for studying the genetic control of PA expression.

Key words: Hybridization, secondary metabolites, defense chemistry, transgressive segregation, covariation

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1. Introduction

The role of hybridization in evolutionary processes including the generation of novel traits, introgression of traits between species, and even speciation has received widespread attention (Stebbins, 1959; Arnold, 1992; Rieseberg and Carney, 1998; Abbott et al, 2009). In recent years it has become apparent that hybridization can lead to the generation of novel molecular and morphological phenotypes (Rieseberg et al, 2003; Kim et al, 2008). Such phenotypes can persist over evolutionary time and can even lead to speciation among hybrid lineages (Seehausen, 2004; Soltis and Soltis, 2009). At the metabolic level, hybridization can impact the diversity of secondary metabolites (SMs) in plants (Orians, 2000). SMs are important for mediating interactions between plants and their environment (Iriti and Faoro, 2009), and the composition of plant SMs can play a role in determining the evolutionary success of populations and species (e.g. Burow et al, 2010).

In the first (or F_1) hybrid generation, most phytochemicals are either expressed at concentrations similar to one of the parents or intermediate to both of the parents (Orians, 2000). However recombination in F_2 and later generation hybrids is expected to increase variation in phytochemical expression among different F_2 genotypes. Transgressive segregation can occur, such that some F_2 genotypes may vary outside the range observed in parental genotypes, and provide key variation upon which selection can act during the process of adaptation (Rieseberg et al, 1999 and 2007). One of the drawbacks of many studies that quantify SM expression by hybrids is that only mean values are reported for each hybrid class (i.e. F_1, F_2, or backcross; e.g. Hallgren et al, 2003; O'Reilly-Wapstra et al, 2005). When genotypes are pooled within classes, transgressive phenotypes may not be identified. Also, many studies fail to carry out replicate measurements from genotypes within parental or hybrid classes and such studies therefore fail to measure and test for genetically controlled variation in SM expression within these classes. In this study, we investigated variation among more than 100 replicated F_2 hybrids, which allowed us to conduct appropriate statistical testing to identify differences between and among hybrid and parental genotypes.

SM accumulation can be influenced by a number of factors including genetics, abiotic factors (such as nutrient and light availability), biotic factors (including competition, herbivory and disease), and interactions between these factors (Lankau and Kliebenstein, 2009; Kirk et al, 2010). However, little is known about the mechanisms behind these complex regulatory systems. Recent work on the genomics and ecology of model and non-model species has started to shed light on the control of SM expression. For example, studies of glucosinolate expression in *Arabidopsis thaliana* have identified four major genetic loci responsible for the expression of 14 different glucosinolates (Kliebenstein, 2009). In addition to the regulatory complexity within individuals, there is considerable variation in SM profiles both within and among plant populations (e.g. Burow et al, 2010). Furthermore, more attention has been paid to SMs in above-ground plant parts than below-ground plant parts, even though the latter is probably equally important to a species' ecology, and there is often interaction or coordination between the expression of SMs in above-ground and below-ground plant tissues (van Dam et al, 2009). Species in the genus *Jacobaea* (syn. *Senecio*, Asteraceae) have been used to investigate the evolutionary basis of SM diversity in plants, because they contain a diverse but structurally related group of alkaloids that play a role in biotic interactions (e.g. Hartmann 1999; Hol and van Veen, 2002; Macel and Vrieling, 2003; Macel et al, 2005; Kowalchuk et al, 2006). Twenty-six pyrrolizidine alkaloids (PAs) have been reported from 24 species of *Senecio* sect. *Jacobaea* (Pelser et al, 2005), although the recent development of more sensitive analytical methods has allowed for the detection of a greater number of structural PA variants in the same species (Joosten et al, 2009, 2010 and Chapter 3). In *Jacobaea* species, all PAs except for senecivernine are derived from senecionine *N*-oxide. Senecionine *N*-oxide is synthesized in the roots, transported to the shoots via the phloem, and diversified into other PA structures (Hartmann and Toppel, 1987, Sander and Hartmann, 1989; Hartmann et al, 1989). Structurally derived PAs are thought to be produced from the precursor senecionine *N*-oxide via a limited number of steps (Hartmann and Dierich, 1998, see a schematic diagram representing putative PA biosynthetic pathways in Fig.S1). Aside from structural diversification, PAs do not undergo any turnover or degradation (Sander and Hartmann, 1989; Hartmann and Dierich, 1998). PAs can occur in plants in two forms: tertiary amine (free base) and *N*-oxide (Rizk, 1991; Wiedenfeld et al, 2008; Chapter 3). The proportion of tertiary amine is different among PAs and between genotypes. In *Jacobaea* plants, the tertiary amine form is usually present among higher proportions in jacobine-like PAs than among senecionine-like and erucifoline-like PAs. However, the mechanisms by which one form is converted to the other are not well understood (Chapter 3).

PA composition and concentration varies greatly between and within *Jacobaea* species (Witte et al, 1992; Macel et al, 2002 and 2004; Pelser et al, 2005). Four different PA chemotypes of *Jacobaea vulgaris* are reported to occur; these include jacobine, erucifoline, mixed and senecionine chemotypes (Witte et al, 1992; Macel et al, 2004). Field studies and controlled bioassays that incorporate herbivores indicate that plant resistance to herbivorous invertebrates is correlated with plant PA concentration and composition (Leiss et al, 2009; Macel and Klinkhamer, 2010). Individual PAs have different deterrent effects on generalist herbivores (Macel et al, 2005), and also have different stimulatory effects on the oviposition of the specialist herbivore *Tyria jacobaeae* (the cinnabar moth; Macel and Vrieling, 2003). Furthermore, free base PAs appear to have different effects on generalist herbivores compared to their corresponding *N*-oxides (van Dam et al, 1995; Macel et al, 2005). These cumulative findings indicate that PA diversity is ecologically important with respect to interactions between plants and herbivores.

Interspecific hybridization is widespread in the S*enecio* genus, including section *Jacobaea* (e.g. Vincent, 1996). For example, hybridization between *Senecio squalidus* and *Senecio vulgaris* led to the origin of three new fertile hybrid taxa, and *S. squalidus* itself is a hybrid species resulting from a cross between *Senecio aethnensis* and *Senecio chrysanthemifolius* (Abbott and Lowe, 2004; James and Abbott, 2005; Abbott et al, 2009). There are many other well-documented cases of hybridization between *Senecio* species (e.g.Beck et al, 1992; Hodalova, 2002; Lopez et al, 2008), including natural hybridization between *J. vulgaris* (formerly *Senecio jacobaea* L.) and *J. aquatica* (formerly *Senecio. aquaticus* L.) which occurs in The Zwanenwater Nature Reserve in The Netherlands (Kirk et al, 2004).

Jacobaea vulgaris (Tansy ragwort or Common ragwort) is native to Europe and west Asia but is invasive in North America, Australia and New Zealand. *Jacobaea aquatica* (Marsh ragwort) is closely related to, but not a sister species of *J. vulgaris* (Pelser et al, 2003). The two species are ecologically distinct. *Jacobaea vulgaris* often occurs in dry, sandy soil with little organic matter and *J. aquatica* is found in wet habitats in soils that are high in organic matter. The two species are attacked by different guilds of herbivorous insects in the field. Different susceptibility to a generalist herbivore has been observed (Kirk et al, 2004 and 2010). Putative hybrids from the Zwanenwater (The Netherlands), initially identified in 1979 based on highly variable and usually intermediate flower and leaf lobe morphology compared to *J. vulgaris* and *J. aquatica*, were confirmed to be hybrids between these two species using molecular genetic markers and PA composition (Kirk et al, 2004). The natural hybrid

population is highly backcrossed with *J. vulgaris*, and F_1 hybrids are uncommon in the natural population (Kirk et al, 2004 and 2005). Different from *J. vulgaris*, *J. aquatica* lacks jacobine-like PAs but is rich in senecionine-like PAs (Kirk et al, 2010). A previous study that characterized PA composition of natural hybrids and artificial F_1 hybrids of the two species showed that PA expression was affected by species and environment interactions (Kirk et al, 2010).

To obtain a hybrid family we selected a *J. vulgaris* genotype of the jacobine-chemotype, which is rich in jacobine-like PAs, and a *J. aquatica* genotype. We established an artificial *J. vulgaris* × *J. aquatica* family, which includes two parental genotypes, two F_1 hybrids, and approximately 100 different F_2 hybrid genotypes. These are all kept in tissue culture and can be reproduced at length. The hybrid system to a great extend overcomes the problem of unavailability of the relevant pure PAs for the study of the effects of individual alkaloids or PA combinations. Kirk et al (2011) reported transgressive segregation of primary and secondary metabolites in the F2 hybrids of this cross using NMR-based metabolomics,

In this study, we aimed to investigate whether hybridization can generate new PA variation in this system and to gain an initial understanding of how PA accumulation is genetically regulated based on the pattern of PA variation. We focused on differences in PA expression among segregating hybrids originating from a single cross between two parental genotypes, and we grew plants under standard conditions to eliminate the effect of environment on PA expression. The methods used in this study differed from those used in previous work in two respects: First, the large numbers of genotypes and replications resulted in a very large sample size; secondly, we measured PAs by LC-MS/MS, which is highly sensitive and can detect the two forms of PAs simultaneously (Joosten et al, 2010). We addressed the following questions: Do F_2 hybrids produce novel PAs? Does any F_2 hybrid genotype show evidence of transgressive variation (over-expression or under-expression) with regard to the concentrations of total PA, a structural group of PAs, or any individual PAs? Does hybridization produce novel PA compositions among F_2 genotypes? Is there covariation in the expression of individual PAs? Are there correlations between the accumulation of PAs in the roots and shoots?

2. Material and Methods

2.1. Study system

Jacobaea vulgaris subs. *dunensis*, *J. aquatica* subs. *aquatica* (parental species, parents), and F_1 and F_2 hybrids of these species were used in this study. *Jacobaea vulgaris* seeds were collected at Meijendel Nature Reserve (52° 7′ 54″ N, 4° 19′ 46″ E, The Netherlands), and *J. aquatica* seeds were collected at the Zwanenwater Reserve (52° 48′ 38″ N, 4° 41′ 7″ E, The Netherlands). Seeds of the two species were sterilized, were germinated in glass vials, and were maintained in tissue culture. Replicate genotypes (clones) from each parental species were subsequently grown in pots in climate rooms (humidity 70%, light 16h at 20°C, dark 8h at 20°C). Before blooming, the potted plants were kept in cold room (humidity 70%, light 8h at 4°C, dark 16h at 4°C) for about 10 weeks to get vernalization. Crosses were performed by rubbing flower heads together (both species are self-incompatible; Kirk et al, 2005 and 2010). Two rayed F_1 offspring were selected from this initial cross, and were reciprocally crossed with each other to produce two sets of offspring. A number of F_1 crosses were made, and we selected the family that produced the greatest number of viable F_2 genotypes. From the selected F_1 cross, we obtained one set of 56 F_2 individuals, and a second set (from the reciprocal cross) of 46 F_2 individuals. The parental, F_1 and F_2 individuals were maintained in tissue culture and were cloned in order to obtain replicate genotypes for the experiments described here. These cloned individuals are referred to as genotypes hereafter. The hybrid status of F_1 and F_2 individuals used in this study was confirmed using AFLP and SNP markers (unpublished).

2.2. Plant growth

We aimed to use six cloned replicates per F_2 genotype and ca. 12 cloned replicates per parental and F_1 genotype, however a few plants died or grew poorly in tissue culture, and were therefore not included in the experiment. Plants were propagated by tissue culture and were potted in 1.3 liter pots filled with 95% sandy soil (collected from Meijendel), 5% potting soil (Slingerland Potgrond, Zoeterwoude, the Netherlands) and 1.5 g l/1 Osmocote slow release fertilizer (Scott®, Scotts Miracle-Gro, Marysville, Ohio, USA; N : P : K = 15 : 9 : 11). Plants were kept in a climate room for six weeks (humidity 70%, light 16h at 20°C, dark 8h at 20°C). In total, we grew more than 600 individual plants including replicates of the two parental, two F_1 and 102 F_2 genotypes.

2.3. Plant harvesting

Plants were harvested after six weeks. Whole plants were gently removed from the potting medium. Shoots were separated from roots with scissors just above the root crown, and roots were rinsed with water. Roots and shoots from each plant were immediately wrapped in a piece of aluminum foil and kept in a cooler with liquid nitrogen until harvesting was completed, then were stored at -80°C until freeze-drying. In total, we harvested the shoots and roots from 609 plants. Each parental and F_1 hybrid genotype was replicated 11 or 12 times. F_2 hybrid genotypes were replicated 3-6 times. In most cases there were six replicates per F_2 genotype; however in a few cases some replicates were lost due to plant death or poor growth. Samples were freeze-dried for one week under vacuum with a collector temperature of -55°C (12-liter Freeze Dry System, Labconco Free Zone®, Labconco Corporation, Kansas City, Missouri,USA). The dry weights of shoots and roots were measured, and plants were ground into fine powder and stored in -20°C until PA extraction.

2.4. Pyrrolizidine alkaloid extraction and analysis

Approximately 10 mg of powdered plant material was extracted with 1 ml 2% formic acid. Heliotrine, monocrotaline and monocrotaline *N*-oxide were added as internal standards to the extraction solvent at a concentration of 1 µg/ ml. The plant extract solution was shaken for 30 minutes. Solid plant material was removed by centrifugation at 720 ×g for 10 min and filtered through a 0.2 µm nylon membrane (Acrodisc 13-mm syringe filter, Pall Life Sciences, Ann Arbor, MI, USA). An aliquot of the filtered solution (25 µl) was diluted with water (975 µl) and injected in the LC-MS/MS system.

A Waters Acquity ultra performance liquid chromatographic (UPLC) system coupled to a Waters Quattro Premier XE tandem mass spectrometer (Waters, Milford, MA, USA) was used for PA analysis. Chromatographic separation was achieved on a Waters Acquity BEH C18 150×2.1 mm, 1.7 µm UPLC column, run with a water/acetonitrile linear gradient containing 6.5 mM ammonia at a flow of 0.4 ml/min. The gradient started at 100% water and during analysis the acetonitrile percentage was raised in 12 min to 50%. The column was kept at 50°C and the injection volume was 10 µl. The MS

system was operated in positive electrospray mode. Data were recorded in multiple monitoring mode (MRM) using two selected precursor ion to product ion transitions per compound. Cone energy was 40 V and collision energy settings were optimized for the individual compounds. In Table 1 an overview is given of the mass spectrometric settings used for the detection of the relevant PAs. The samples were run in a randomized order divided over 5 series. For each compound the sum of the two peak areas was normalized against the peak area of the internal standard heliotrine. Quantification was performed against a standard solution (100 µg/ l) of the PAs in a diluted extract of *Tanacetum vulgare* (Tansy). The extract of *T. vulgare* material was prepared in the same way as the other extracts and was used to mimic a PA-free plant extract. The standard solution was injected every 30 samples, and the averaged response of each compound was used for quantification. Seventeen individual PA standards (detail of the source of the standards in Chapter 3, 5) were available for this study, representing over 80% of the total amount of PAs present in the majority of plants extracts. For those compounds for which no reference standard was available, a semi-quantitative (indicative) value could be obtained by comparison with the most closely related analogue (e.g, an isomer). Identification of these PAs was based on their retention time, molecular mass, fragmentation pattern and on comparison with PA standards and/or literature data. Data processing was conducted with Masslynx 4.1 (Waters Corporation, Milford, MA, USA).

2.5. Data analysis

We checked for maternal effects on both quantitative and qualitative variation with regard to F_2 genotypes from different maternal F_1 parents within the reciprocal cross (data not shown). Since no significant maternal effects were found, F_2 genotypes from both maternal parents were pooled for the analysis.

2.5. 1. Analysis of PA qualitative variation

The genotype-dependent presence of florosenine, floridanine and doronine in the roots and shoots was tested using binomial general linear models in which PA concentration values were coded as either 0 (absent) or 1 (present) and genotype was designated as the fixed factor. We carried out qualitative analyses incorporating these three PAs because they were the only PAs that were absent in some samples. All other PAs were always present.

2.5. 2. Analysis of PA quantitative variation

We classified the PAs identified in this study into four types according to their structural characteristics and bio-synthetic pathways (see Figs. S1-2; Pelser et al, 2005): senecionine-like PAs, jacobine-like PAs, erucifoline-like PAs and otosenine-like PAs (Table 1). Senecivernine and senkirkine were not grouped with any other PAs by Pelser et al (2005). However based on the experimental data obtained in our PA measurements, senecivernine expression was closely correlated with the expression of senecionine-like PAs, and senkirkine expression was similarly correlated with that of otosenine-like PAs. Senecivernine and senkirkine were therefore grouped respectively with senecionine-like PAs and otosenine-like PAs for the purposes of analysis.

We used ANOVAs to test whether PA quantities in roots and/or shoots were dependent on genotype. We defined each PA as a separate dependent variable. We also used ANOVAs to test whether the four structural groups of PAs, free bases, *N*-oxides, and total PA were dependent on genotype. The data were log-transformed. We tested for normal distribution and homogeneity of the variance using the residuals from the models. Differences between the hybrids and parental genotypes were evaluated from the data in regression coefficient matrices of the models. In each matrix, the estimated coefficient of a hybrid indicated whether it had a lower or higher amount of PA than one of the

Table 1. PAs detected in *Jacobaea aquatica*, *Jacobaea vulgaris* and hybrids. Retention times and selected mass spectrometric conditions are given.

Group	PA	Code	Retention time (min)	Precursor mass (m/z)	Fragment mass 1; 2 (m/z)	Collision energy 1; 2 (eV)	Standard used for quantification
Senecionine-like PAs (simple senecionine-related derivatives)	senecionine	sn	9.93	336.2	94.0; 120.0	40; 30	sn
	senecionine *N*-oxide	snox	6.97	352.2	94.0; 120.0	40; 30	snox
	integerrimine	ir	9.72	336.2	94.0; 120.0	40; 30	ir
	integerrimine *N*-oxide	irox	6.83	352.2	94.0; 120.0	40; 30	irox
	retrorsine	rt	8.49	352.2	94.0; 120.0	40; 30	rt
	retrorsine *N*-oxide	rtox	6.01	368.2	94.0; 120.0	40; 30	rtox
	usaramine	us	8.29	352.2	94.0; 120.0	40; 30	rt
	usaramine *N*-oxide	usox	5.89	368.2	94.0; 120.0	40; 30	rtox
	riddelliine	rd	7.91	350.2	94.0; 138.0	40; 30	rd
	riddelliine *N*-oxide	rdox	5.48	366.2	94.0; 118.0	40; 30	rdox
	seneciphylline	sp	9.16	334.2	94.0; 120.0	40; 30	sp
	seneciphylline *N*-oxide	spox	6.36	350.2	94.0; 138.0	40; 30	spox
	spartioidine	st	8.96	334.2	120.0; 138.0	30; 30	sp
	spartioidine *N*-oxide	stox	6.36	350.2	94.0; 138.0	40; 30	spox
	acetylseneciphylline	acsp	11.80	376.2	120.0; 138.0	30; 30	acsp
	acetylseneciphylline *N*-oxide	acspox	8.86	392.2	94.0; 118.0	40; 30	acspox
	senecivernine	sv	10.09	336.2	94.0; 120.0	40; 30	ir
Jacobine-like PAs (jacobine-related derivatives)	jacobine	jb	7.89	352.2	120.0; 155.0	30; 30	jb
	jacobine *N*-oxide	jbox	5.49	368.2	120.0; 296.0	30; 25	jbox
	jacoline	jl	6.13	370.2	94.0; 138.0	40; 30	jb
	jacoline *N*-oxide	jlox	4.39	386.2	94.0; 120.0	40; 30	jbox
	jaconine	jn	8.75	388.2	94.0; 120.0	40; 30	jb
	jaconine *N*-oxide	jnox	5.77	404.2	94.0; 138.0	40; 30	jbox
	jacozine	jz	7.23	350.2	94.0; 138.0	40; 30	jb
	jacozine *N*-oxide	jzox	5.11	366.2	94.0; 118.0	40; 30	jbox
	dehydrojaconine	dhjn	7.86	386.2	94.0; 120.0	40; 30	jb
Erucifoline-like PAs (erucifoline-related derivatives)	erucifoline	er	7.56	350.2	94.0; 120.0	40; 30	er
	erucifoline *N*-oxide	erox	4.80	366.2	94.0; 118.0	40; 30	erox
	acetylerucifoline	acer	10.18	392.2	94.0; 118.0	40; 30	er
	acetylerucifoline *N*-oxide	acerox	7.17	408.2	94.0; 120.0	40; 30	erox
Otosenine-like PAs (otosenine-related derivatives)	senkirkine	sk	7.31	366.2	122.0; 168.0	30; 25	sk
	otosenine	ot	5.60	382.2	122.0; 168.0	30; 25	sk
	onetine	one	4.35	400.2	122.0; 168.0	30; 30	sk
	desacetyldoronine	desdor	6.26	418.2	122.0; 168.0	30; 30	sk
	florosenine	fs	8.35	424.2	122.0; 168.0	35; 30	sk
	floridanine	fd	6.79	442.2	122.0; 168.0	30; 30	sk
	doronine	dor	9.01	460.2	122.0; 168.0	30; 30	sk

parents, and the *P*-value showed whether the difference was significant (Crawley, 2005). The hybrids were compared to each of the two parents separately.

There were a number of variables (see details in Table S3) that did not meet the assumptions for a linear model. We tested among-genotype differences in these variables using Kruskal-Wallis tests for which PA concentrations were defined as independent variables and genotype was defined as the factor. The data were log-transformed to achieve homogeneity of the variance among genotypes. Differences between hybrid and parental genotypes were evaluated using multiple comparisons after Kruskal-Wallis tests, for which either of the parents was defined as the control (Giraudoux, 2010).

The type of quantitative PA variation (in hybrids compared to parents) was classified as follows: under-expression (U, concentration in hybrid significantly less than that of both parents); dominant to the parent with lower expression (Dl, concentration in hybrid not different from the parent with lower expression and significantly different from the other parent); intermediate to the parents (Im, concentration in hybrid intermediate to but significantly different from both parents); dominant to the parent with higher expression (Dh, concentration in hybrid not different from the parent with higher expression and significantly different from the other parent); over-expression (O, concentration in hybrid significantly greater than that of both parents); not different from the parents (ND, not significantly different from either parent).

2.5. 3. Analysis of PA composition

Differences in PA composition were evaluated using relative concentrations of individual PAs. The relative concentration was calculated as follows: (absolute concentration of an individual PA or a group of PAs) / (total PA concentration) × 100. The relative concentration data were not normally distributed and the variances among the genotypes were not homogeneous. We therefore tested for differences in relative PA concentration among genotypes using Kruskal-Wallis tests and non-parametric multiple comparisons (Giraudoux, 2010).

Differences in PA composition among genotypes and between the shoots and roots were tested using an Adonis test, which is a non-parametric MANOVA (Oksanen et al, 2010). Genotype and plant part (shoots or roots) were defined as factor variables. We visualized variation in PA composition using a non-metric multidimensional scaling (NMDS) method, which is analogous to PCA or multidimensional scaling (MDS) but without distribution assumptions (Goslee and Urban, 2007). As in a PCA or MDS plot, each point in the NMDS plot represents an individual sample, and points that are close together indicate that those samples have similar PA compositions. NMDS can avoid the arch and compressed pattern that occurs in PCA when data includes samples that have few components in common (Quinn and Keough, 2002).

2.5. 4. Cluster and correlation analysis

A hierarchical cluster analysis of individual PAs in shoots and roots was carried out to identify similarities in the expression of different PAs. The data used in this analysis were log-transformed absolute PA concentrations. The hierarchical cluster analysis was carried out using the likelihood linkage analysis method (Kojadinovic, 2010). We tested for correlations between PA concentrations in the shoots and roots using Spearman correlation tests (on absolute concentrations). *P*-values were adjusted for multiple comparisons using sequential Bonferroni methods.

All analyses were conducted in R version 2.10.0 (R Development Core Team, 2009).

3. Results

3.1. PA qualitative variation

In total, we detected 37 PAs in the shoots and roots of the parents, F_1 and F_2 hybrids. We classified each PA into one of four structural groups: senecionine-like PAs, jacobine-like PAs, erucifoline-like PAs or otosenine-like PAs (Table 1). Otosenine-like PAs do not occur as *N*-oxides. PAs of other types were present and detected in both forms, except for dehydrojaconine and senecivernine, which were only detected in the free base form.

Most parental PAs were always present in the offspring, though some only in trace amounts (< 0.1µg/g DW=dry weight). Three PAs, florosenine, floridanine and doronine, were present in *J. aquatica* shoots, but were absent in *J. vulgaris* shoots and were absent (or present in trace amounts) in the roots of both parents. These three PAs were present in the shoots and roots of the two F_1 hybrids. They were absent in the shoots and/or roots of some F_2 genotypes, but were present in much higher concentrations in some F_2 plants compared to the parents (Table 2, Table S1-2). The presence of all three of these PAs was genotype dependent both in the shoots and roots (shoots and roots tested separately, in all cases: $df = 105$; $\chi^2 > 600$, $P < 0.01$).

Table 2. Qualitative variation of three otosenine-like PAs in the roots and shoots of two F_1 and 102 F_2 hybrids between *Jacobaea aquatica* and *Jacobaea vulgaris*. All other PAs reported in this study were always present in parents, F_1 hybrids, and F_2 hybrids.

PAs		*J. aquatica*	*J. vulgaris*	F_1-A	F_1-B	F_2 Absent	F_2 Present
florosenine	roots	Trace	Present	Present	Present	32	70
	shoots	Present	Absent	Present	Present	28	74
floridanine	roots	Absent	Absent	Present	Present	38	64
	shoots	Present	Absent	Present	Present	37	65
doronine	roots	Trace	Absent	Present	Present	37	65
	shoots	Present	Absent	Present	Present	40	62

Numbers indicate the number of F_2 genotypes in which a particular PAs was absent or present. If a certain PA was present in the roots or shoots of a single replicate, we scored that PA as present in that genotype. If the PA was not found in any of the replicates, it was regarded absent in the genotype. Trace indicates concentrations less than 0.1µg /g DW.

3.2. PA quantitative variation

We analyzed quantitative variation in the concentration of 34 individual PAs (excluding florosenine, floridanine, and doronine), the sum concentrations of the four PA groups (florosenine, floridanine, and doronine were included in otosenine group), the sum concentration of free bases and *N*-oxides, and total PA concentration. All variables were genotype dependent (ANOVA or KW test; separately for shoots and roots; in all cases: $df = 105$; $P < 0.01$).

Jacobaea aquatica had lower total PA concentration than *J. vulgaris* in shoots. Both of the F_1 genotypes were intermediate to the parents. F_2 genotypes were on average intermediate to the parents as well. However, a 20-fold difference in genotypic mean total PA concentration (334.0-6835.0 µg/g DW) was observed among F_2 hybrid genotypes (Fig.1 and Table S1).

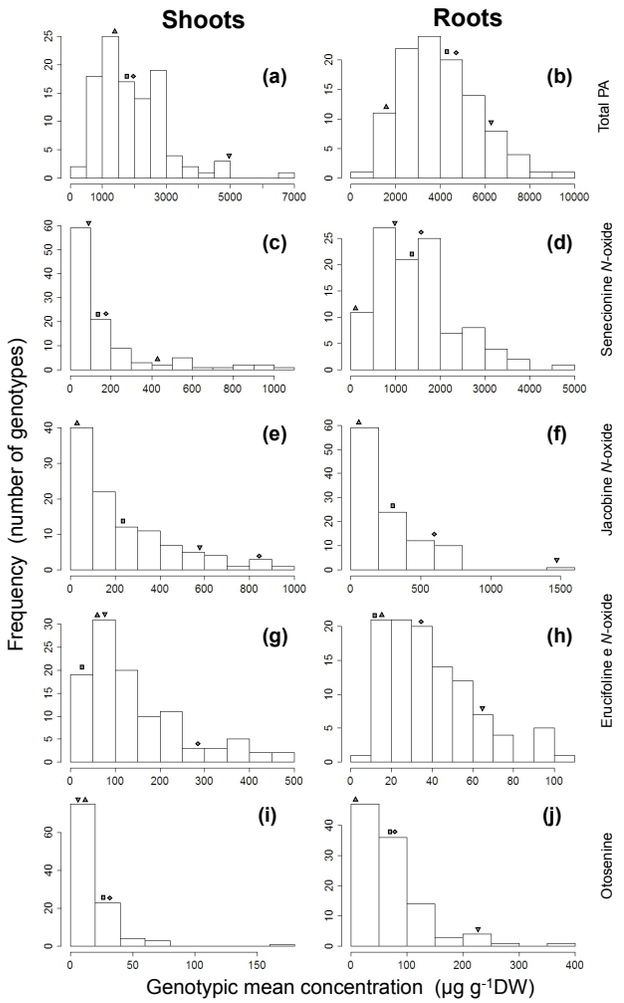

Fig.1 Frequency distribution of genotypic mean concentrations (µg/g DW) of total PA, senecionine *N*-oxide, jacobine *N*-oxide, erucifoline *N*-oxide and otosenine in the shoots and roots of the 102 F_2 hybrid genotypes between *J. aquatica* and *J. vulgaris*. The positions of the symbols above the bars indicate genotypic mean values for the two parental and the two F_1 genotypes. ▲ = *J. aquatica*, ▼= *J. vulgaris*; ■ = F_1-A; ◆ = F_1-B. The genotypic mean concentration is the average value of the 3-6 replicates from the same genotype.

There was also great variation in the quantities of particular groups of PAs and individual PAs (Figs 1 S3 and Table S1). In F_2 hybrid shoots, transgressive segregation (statistically significant under-expression or over-expression) of PA expression occurred in 7.5% of cases for concentrations of individual PAs and also in 7.5% of cases for concentrations of PA groups or total PA concentration (Fig.2 and Table S3). Among the F_2 hybrids, 14 genotypes had significantly lower total PA concentration compared to the parents, and no F_2 genotypes had significantly higher total PA concentration. Otosenine-like PAs (group sum) were overexpressed in the shoot of one F_2 hybrid genotype, as a result of the over-expression of desacetyldoronine and otosenine. Over-expression of erucifoline-like PAs (group sum), erucifoline, and its *N*-oxide was observed in some F_2 hybrids. Over-expression of several minor PAs,

including riddelliine, riddelliine *N*-oxide and jacozine *N*-oxide occurred in a few F_2 genotypes (Fig.2 and Table S3).

Similar patterns of PA expression variation occurred in hybrid roots. Extremely high or low concentrations of individual PAs only occurred in 6.2% of all tests. Some minor PAs such as retrorsine, retrorsine *N*-oxide, riddelliine, seneciphylline, acetylerucifoline and acetylerucifoline *N*-oxide were overexpressed in a few F_2 genotypes. Transgressive concentrations of PA groups and transgressive total PA concentration were rarer (only 0.7% across tests including PA groups and total PA concentration) in the roots compared to the shoots (Fig 2 and Table S3).

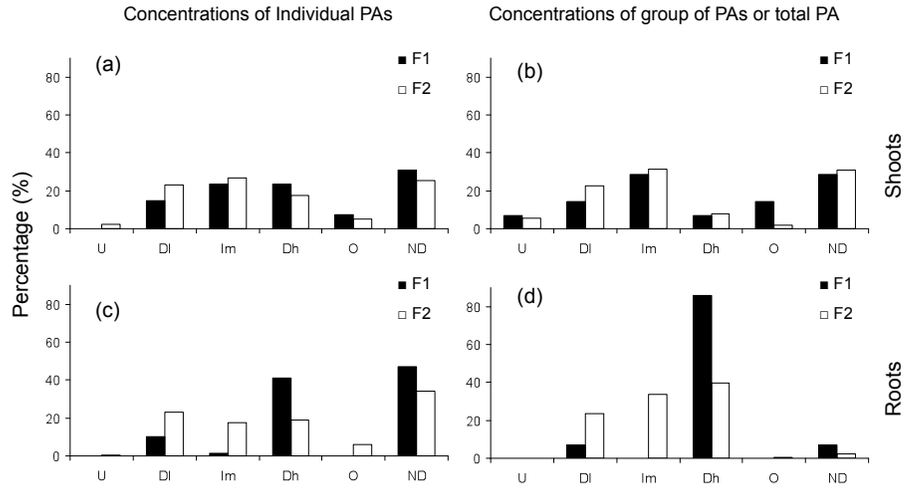

Fig.2 Classification of PA quantitative variation in the shoots and roots of two F_1 and 102 F_2 hybrids relative to the parental genotypes. Hybrid genotypes were classified into six types according to expression of a individual PA, group of PAs or total PA: U (under-expression, significantly less than that of both parents); Dl (dominant to the parent with lower expression, not different from the parent with lower expression and significantly different from the other parent); intermediate to the parents (Im, intermediate to but significantly different from both parents); Dh (dominant to the parent with higher expression, not different from the parent with higher expression and significantly different from the other parent); O (over-expression, significantly greater than that of both parents); ND (not significantly different from the parents). The graphs show percentage of hybrids divided over the different types. See details in Table S3

3.3. Variation in PA composition

PA composition differed in the shoots of the two parental genotypes. Senecionine-like PAs were dominant in *J. aquatica*, and jacobine-like PAs were dominant in *J. vulgaris*. In the roots of *J. aquatica*, more than 96% of the total PA belonged to the senecionine group. In contrast to the shoots, senecionine-like PAs were also dominant in the roots of *J. vulgaris*, and comprised approximately 60% of the total PA, while jacobine-like PAs comprised about 30% and otosenine-like PAs comprised 5%. Erucifoline-like PAs were found only in low concentrations (Fig.3a-d).

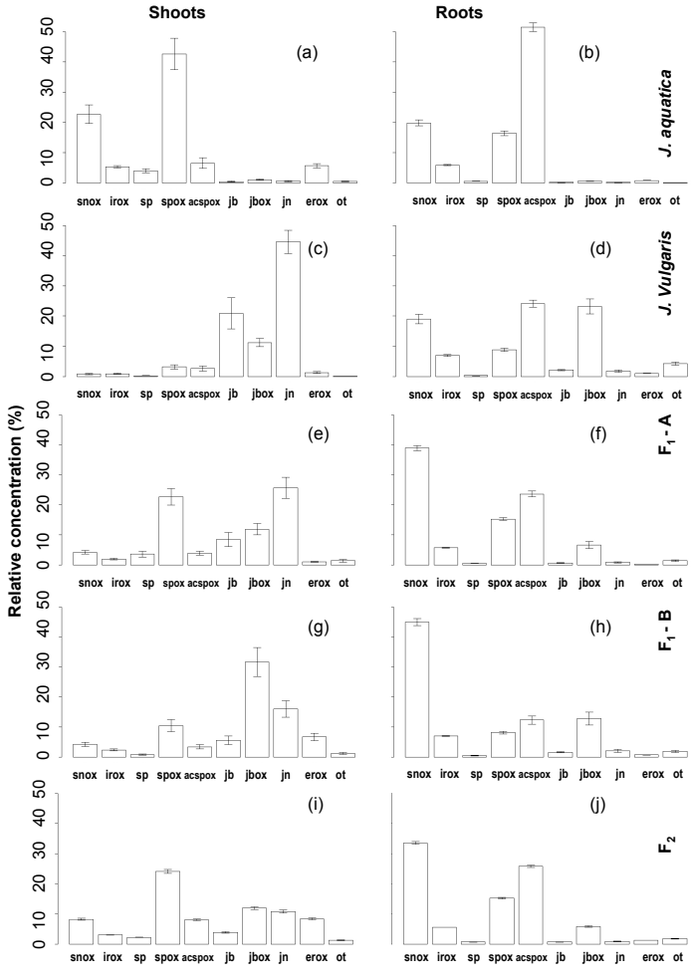

Fig.3 Relative concentrations of major PAs in the shoots and roots of *J. aquatica*, *J. vulgaris*, F_1 and F_2 hybrids. Relative concentrations represent the percentages of total PA concentration in a sample. The PAs shown in the graphs are the 10 PAs with the highest relative concentrations across all samples. Error bars are standard errors. The graph of F_2 is based on the mean relative concentrations of individual PAs for all samples of the F_2 genotypes and the other graphs represent individual samples from the same genotype. *J. aquatica*, one genotype, 12 replicates; *J. vulgaris*, one genotype, 12 replicates; F_1-A, one genotype, 11 replicates; F_1-B, one genotype, 12 replicates; F_2, 102 genotypes, 3-6 replicates per genotype. Abbreviations for PAs are defined in Table 1.

The shoots of the two F_1 hybrids showed a mixed pattern compared to the parents; concentrations of senecionine-like and jacobine-like PAs were approximately equal. The roots of F_1 hybrids contained a greater variety of PAs than those of *J. aquatica*. They contained more than 10% jacobine-like PAs, and also contained some other PAs including erucifoline and otosenine. However the relative concentration of senecionine-like PAs remained high at approximately 80% or more (Fig.3e-h). The shoots and roots of F_2 hybrids on average showed patterns similar to the F_1 hybrids (Fig.3i,j), but individual F_2 hybrids showed variable patterns (Fig.S4).

Differences in PA composition between genotypes were significant in both shoots and roots, and differences between the shoots and roots were also significant (two factor Adonis test; genotype: $df = 105$, $r^2 = 0.31$, $P = 0.01$; plant part: $df = 1$, $r^2 = 0.36$, $P = 0.01$). The relative concentrations of major PAs and of PA groups were genotype dependent (KW test; in all cases: $df = 105$; $P < 0.01$). Shoots tended to contain greater relative concentrations of jacobine-like PAs than roots, while roots had higher relative concentrations of senecionine-like PAs than shoots. The shoot and root samples could therefore be differentiated into two groups with regard to PA composition (Fig.S4).

3.4. Covariation between individual PAs and shoot/root correlations

We investigated correlations between individual PAs both in the shoots and in the roots. Hierarchical cluster analysis (HCA) was used to visualize the covariation between PAs. Based on the clustering results, the PAs in the shoots could be divided into four groups. Interestingly, these groups correspond to the structural groups shown in Table 1, such that PAs from the same structural group clustered together (see structural groups in Table 1). However, there were some exceptions. Usaramine, spartiodine and their corresponding *N*-oxides are senecionine-like PAs but were not clustered with other senecionine-like PAs. Also, jacozine *N*-oxide clustered with erucifoline-like PAs instead of jacobine-like PAs (Fig.4a). Furthermore, we found that the free base form of each PA often clustered with its corresponding *N*-oxide (Fig.4a, Table S4). A similar pattern was found with regard to the cluster analysis of the PA concentrations in the roots (Fig.4b).

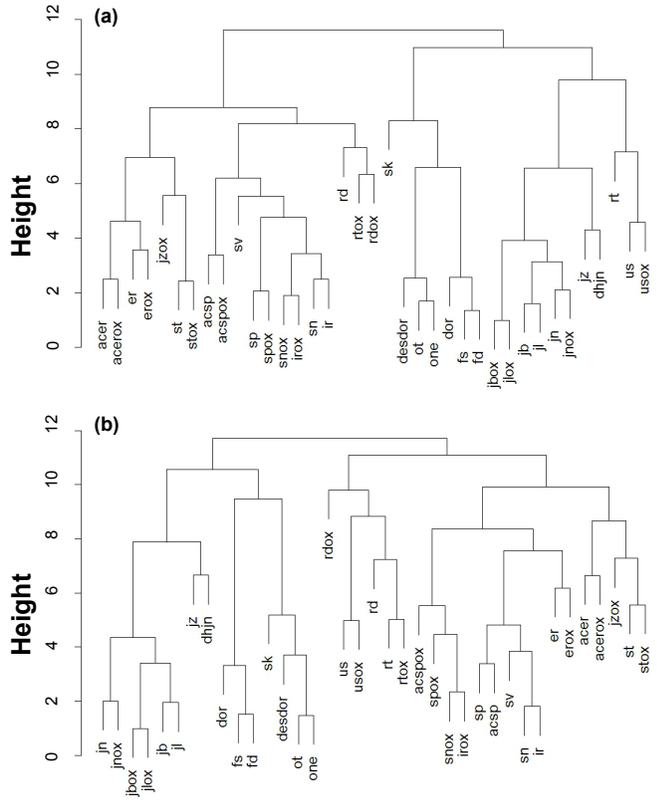

Fig.4 Hierarchical clusters of individual PAs in shoots (a) and roots (b) of *J. aquatica*, *J. vulgaris*, F_1 and F_2 hybrids. The data used in this analysis were the log-transformed absolute concentrations of individual PAs. *J. aquatica*, one genotype, 12 replicates; *J. vulgaris*, one genotype, 12 replicates; F_1-A, one genotype, 11 replicates; F_1-B, one genotype, 12 replicates; F_2, 102 genotypes, 3-6 replicates per genotype. Abbreviations for PAs are defined in Table 1.

We compared the concentration of individual PAs, PA groups and total PA between shoots and roots. Concentrations of all individual PAs were significantly positively correlated between roots and shoots. Consequently, the concentrations of total PA and of all four groups were also correlated between these two tissues (Table 3).

Table 3. Spearman rank correlations between PA concentration in shoots and roots of *Jacobaea aquatic* (one genotype), *Jacobaea vulgaris* (one genotype), F_1 hybrids (two genotypes) and F_2 hybrids (102 genotypes). In all cases: $df = 607$, $P < 0.01$.

Group	PA	r_s	PA	r_s
Senecionine-like PAs	senecionine	0.53	senecionine *N*-oxide	0.42
	intergerrimine	0.58	intergerrimine *N*-oxide	0.51
	retrorsine	0.41	retrorsine *N*-oxide	0.44
	usaramine	0.54	usaramine *N*-oxide	0.80
	riddelliine	0.22	riddelliine *N*-oxide	0.29
	seneciphylline	0.49	seneciphylline *N*-oxide	0.45
	spartiodine	0.54	spartiodine *N*-oxide	0.60
	acetylseneciphylline	0.54	acetylseneciphylline *N*-oxide	0.40
	senecivernine	0.40		
Jacobine-like PAs	jacobine	0.77	jacobine *N*-oxide	0.83
	jacoline	0.82	jacoline *N*-oxide	0.85
	jaconine	0.83	jaconine *N*-oxide	0.83
	jacozine	0.49	jacozine *N*-oxide	0.66
	dehydrojaconine	0.65		
Erucifoline-like PAs	erucifoline	0.50	erucifoline *N*-oxide	0.46
	acetylerucifoline	0.24	acetylerucifoline *N*-oxide	0.38
Otosenine-like PAs	senkirkine	0.35	florosenine	0.77
	otosenine	0.52	floridanine	0.74
	onetine	0.51	doronine	0.78
	desacetyldoronine	0.61		
Sum	PA free bases	0.57	PA *N*-oxides	0.46
	senecionine-like PAs	0.44	jacobine-like PAs	0.86
	erucifoline-like PAs	0.50	otosenine-like PAs	0.49
	Total PA	0.55		

4. Discussion

4. 1. Novelty resulting from hybridization

In agreement with our expectations, we found that some F_2 hybrid genotypes exhibited extreme expression of some PAs, and novel patterns of overall PA composition. We found evidence for qualitative novelty: three acetylated otosenine-like PAs (florosenine, floridanine and doronine) were present in the roots of F_1 and some F_2 genotypes, but never or only in trace amounts in the roots of the parents, although all three PAs were present in the shoots of *J. aquatica* (Table S1-2). Florosenine was also reported to be novel to F_1 hybrids in a recent study by Kirk et al (2010), although the detection method used by these authors was less sensitive than that used in this study. The expression of a parental SM in novel tissues can lead to new ecological and evolutionary consequences. For example, PAs have been shown to have different effects on the growth of root-associated micro-organisms (Kowalchuk et al, 2006), and the addition of a novel compound in the roots of hybrids might impact interactions with symbiotic or pathogenic microbes.

Some otosenine-like PAs such as desacetyldoronine were overexpressed in the shoots of some F_2 hybrids, and in 10 F_2 hybrids this structural group comprised more than 20% of the total PA present. To our knowledge, otosenine-like PAs have not been previously reported as a major component of the bouquet of PAs in *J. vulgaris* or *J. aquatica*. In addition, overall PA compositions were different in some F_2 hybrids genotypes compared to the parents. The two parental genotypes were well separated according to the NMDS analysis, and differed especially with regard to the relative amount of senecionine-like and jacobine-like PAs in shoots. Many F_2 hybrid genotypes showed PA compositions that were intermediate to those of the parental genotypes (Fig.S4). However, some F_2 hybrid shoots contained a higher relative proportion of erucifoline-like PAs. These F_2 hybrids showed different patterns than those found in the shoots of either parental genotype, in which jacobine-like PAs or senecionine- like PAs were dominant. PAs can have individual effects on aboveground herbivores, or synergistic effects that depend on interactions between multiple PAs within a bouquet (Macel et al, 2005). The ecological role of erucifoline-like PAs is not well understood, but alteration of aboveground PA composition might have implications in terms of susceptibility to generalist and specialist herbivores. Novelty in PA composition among F_2 genotypes illustrates that hybridization might increase the diversity of PA expression within the *Jacobaea* genus. It is also possible that altered PA expression can affect the fitness of natural hybrids, and can in turn mediate population dynamics within natural hybrid populations. These are interesting avenues for further research.

4.2. Differences between shoots and roots

Some interesting differences between PA compositions in the shoots and roots were observed. Generally, shoots contained higher proportions of jacobine- and erucifoline-like PAs and lower proportions of senecionine and otosenine-like PAs compared to roots (Fig 3, S4 and Table S1-2). Moreover, shoots contained greater proportions of biosynthetically derived PAs than the roots (Fig.S4), while the roots contained higher total PA concentrations (Fig 1, S3 and Table S1-2). The mechanisms by which these patterns are established are not yet clear. In another study, a few *J. vulgaris* genotypes derived from natural populations also showed similar patterns (Joosten et al, 2009). However, the ecological implications of different PA compositions and concentrations in roots and shoots remain uncertain. Recent work has shown that jacobine-like PAs are relatively more important than other PA groups for mediating interactions between *Jacobaea* plants and an aboveground generalist herbivore (Western flower thrips; Leiss et al, 2009; Chapter 5; but also see Kowalchuk et al, 2006). If jacobine-like PAs are more important in mediating above-ground interactions than below-ground interactions, it is logical that they should be sequestered to a great extent in above-ground plant parts. Otosenine-like PAs generally accumulate more in the roots (Table S1-2, Fig 3, S3). However, the role of otosenine-like PAs in mediating below-ground interactions has never been investigated.

4.3. Variation patterns and their implications for genetic regulation and biosynthesis

Previous studies have shown that genes that code for the presence of SMs usually have a dominant mode of inheritance: if one or both of the parents produce a particular metabolite, hybrids almost always produced it (Rieseberg and Ellstrand, 1993; Orians, 2000). This was also the case in our study with regard to the expression of PAs in *Jacobaea* hybrids; F_1 and F_2 hybrids always produced all PAs found in the parental individuals. Quantitative variation of SM expression followed a pattern of

continuous variation, which suggests that concentrations of individual PAs and of structural groups are controlled by multiple genes. These genes may include loci coding for the enzymes involved in biosynthetic pathway and/or regulatory genes. The interaction between such genes may show dominant, over-dominant, recessive, additive, or epistatic effects on PA expression, however the number of loci involved in PA diversification and accumulation and their modes of action and interaction cannot be elucidated based on the results of this study. QTL analysis of PA expression will allow us to investigate such genetic effects, and to identify interactions between loci.

We observed that expression of PAs within structural groups was correlated (Fig.4 and Table S4), while PAs from different structural groups (except senecionine-like and erucifoline-like PAs) showed greater independence. This pattern appeared both in the shoots and roots (Fig.4, and Table S4). This suggests that the up- or down-regulation of enzymatic pathways involved in the biosynthesis of derived structural groups (ie. erucifoline-, jacobine- and otosenine-like PAs) may be active processes, but diversification within structural groups is more passive. In other words, once the pathway leading to the biosynthesis of PAs from a particular structural group (e.g. jacobine like PAs) is turned on, several different PAs from within that group (jacobine, jacozine, jacoline, etc) are synthesized in a codependent manner. Furthermore, the high correlation between the PA free bases and their corresponding *N*-oxides indicates that the conversion of PAs between the two forms may be a passive, concentration-dependent, and PA-structurally specific process (also see Chapter 3).

In spite of the differences in PA compositions between shoots and roots, these two tissues showed positive correlations with regard to the absolute concentrations of PAs. This pattern can be explained by processes of PA synthesis and accumulation in *Jacobaea* (*Senecio*) plants. The concentration of a particular PA in the shoots and/or roots is determined by a number of steps: (1) synthesis of the backbone structure senecionine *N*-oxide, which occurs mostly in the roots of *Jacobaea* (*Senecio*) plants, (2) structural transformation, which occurs primarily in the shoots, and (3) translocation and storage of PAs. Root-to-shoot translocation of PAs occurs exclusively via the phloem. Once they are synthesized, PAs do not undergo any degradation or turnover. They are slowly but steadily distributed within the plant (reviewed by Hartmann and Ober, 2000). Therefore, it is not surprising that there were positive and highly significant correlations between PA concentrations in the shoots and roots.

In conclusion, understanding the mechanisms and consequences of such patterns of PA variation may provide fascinating clues with regard to biosynthetic pathways, evolutionary constraints, and the ecological role of these SMs. Furthermore, the hybrid system described in this study is a useful tool for understanding the ecological role of PA variation, because a great diversity of PA patterns is found among segregating hybrids. We detected 37 individual PAs in above- and below-ground plant parts, including both free base and *N*-oxide forms of many PAs, using LC-MS/MS. We found qualitative and quantitative differences in the patterns of PA variation in segregating hybrids compared to parental genotypes. Moreover, we revealed that PAs from within structural groups covary, and there are significant correlations between the accumulation of PAs in the shoots and roots.

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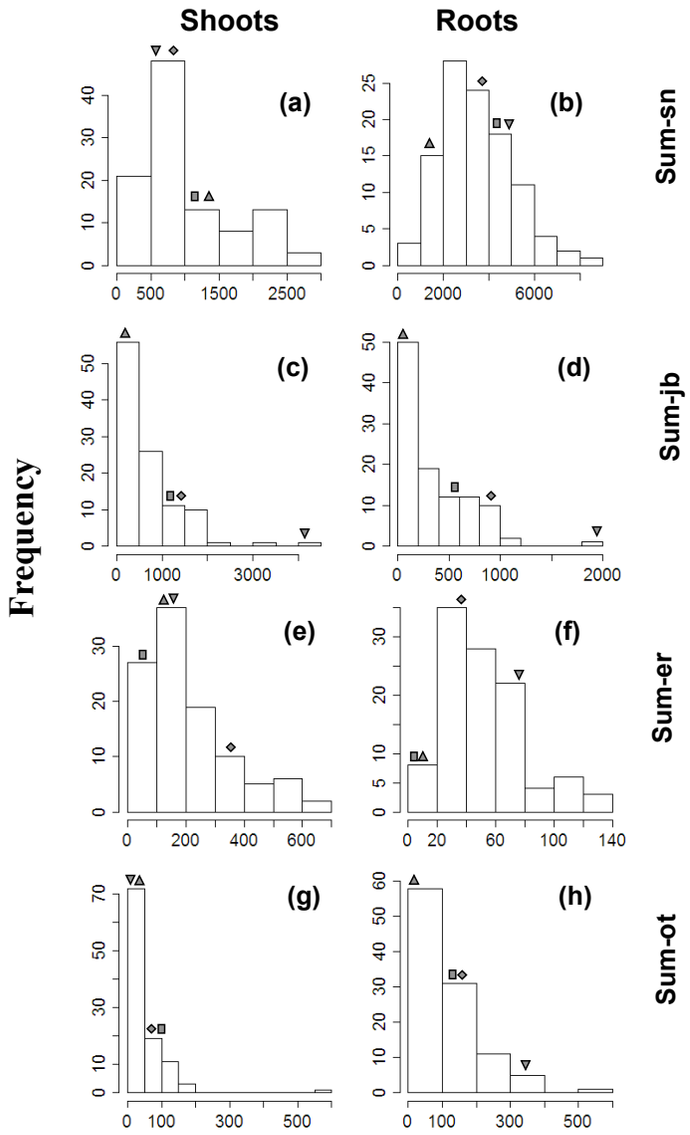
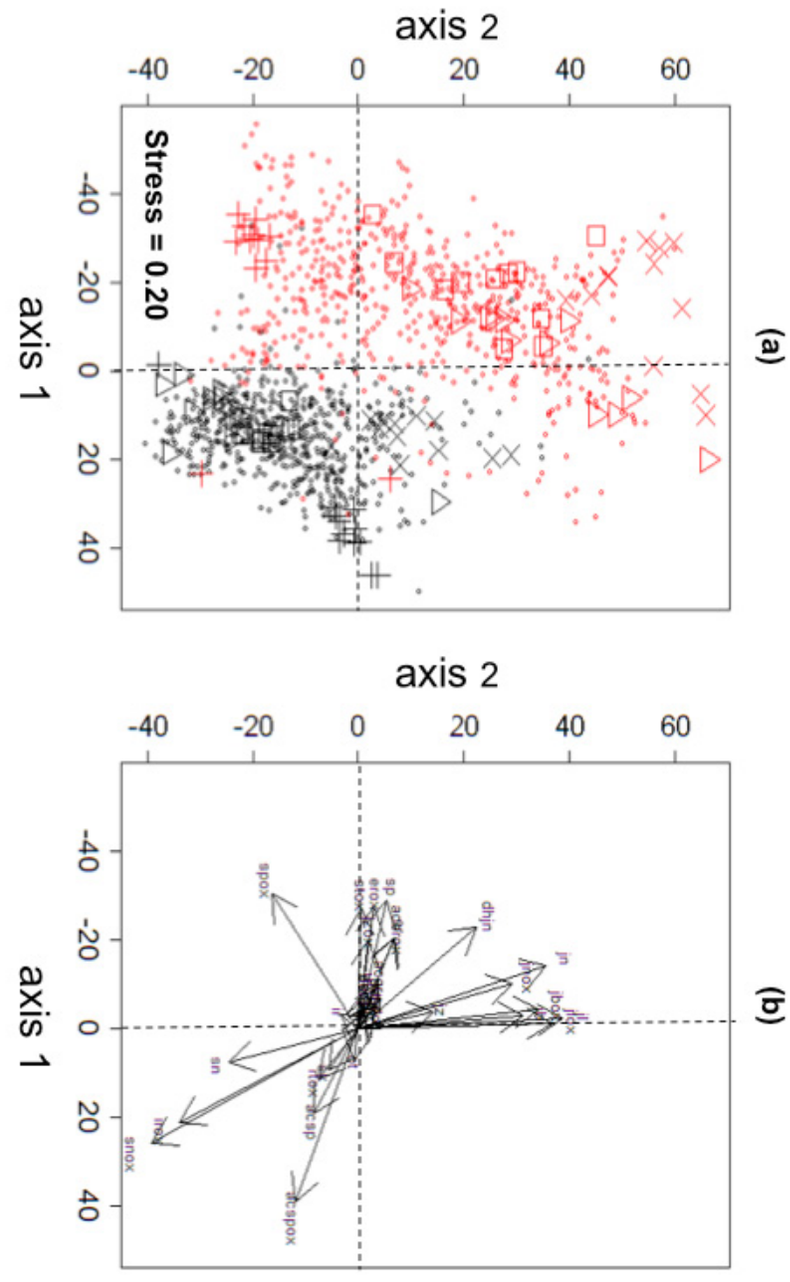
Supplementary material

- **Fig.S1-2** are Appendix 1-2 at the end of this thesis



- **Fig.S3** Frequency distribution of genotypic mean concentrations (µg/g DW) of PAs from four structural groups in the shoots and roots of 102 F_2 hybrid genotypes between *Jacobaea aquatica* and *Jacobaea vulgaris*. The positions of the symbols above the bars indicate approximate values for parental and F_1 genotypes. ▲ = *J. aquatica*, ▼= *J. vulgaris*; ■ = F_1-A; ◆ = F_1-B. The genotype-specific concentration is the average value for the 3-6 replicates from the same genotype. Sum-sn: the sum of all senecionine-like PAs. Sum-jb: the sum of all jacobine-like PAs. Sum-er: the sum of all erucifoline-like PAs. Sum-ot: the sum of all otosenine-like PAs, including florosenine, floridanine, and doronine.



- **Fig.S4** PA composition in the shoots and roots plotted by two-dimension nonparametric multidimensional scaling (NMDS; a) and the loadings (b). Analysis was based on relative concentration of all individual PAs. Cross: *Jacobaea aquatica*, one genotype, 12 replicates; rotated cross: *Jacobaea vulgaris*, One genotype, 12 replicates; square, F_1-A, one genotype, 11 replicates; Diamond, F_1-B, one genotype, 12 replicates; circle, F_2, 102 genotype, 3-6 replicates per genotype. Red symbols represent shoots and black symbols represent roots. Abbreviations for PAs are defined in Table 1.

- **Table S1**. PA concentrations (µg g^{-1} DW) in the shoots of *J. aquatic* (one genotype), *J. vulgaris* (one genotype), F_1 hybrids (two genotypes) and F_2 hybrids (102 genotypes)

PAs	*J. aquatica*		*J. vulgaris*		F_1-A		F_1-B		F_2			
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Min	Max
senecionine	28.8	10.1	2.7	0.6	12.6	5.1	5.7	1.2	10.5	0.8	0.3	65.4
senecionine *N*-oxide	413.2	152.4	45.5	13.0	104.7	31.5	123.1	22.2	177.6	14.8	4.9	1044.5
integerrimine	5.2	1.2	3.1	0.7	5.2	2.1	2.9	0.7	3.2	0.2	0.3	12.0
integerrimine *N*-oxide	86.2	19.4	52.0	13.7	51.8	18.9	78.3	18.4	62.0	3.5	4.7	235.9
retrorsine	0.6	0.1	4.2	1.8	0.9	0.2	1.0	0.3	1.0	0.1	0.3	3.7
retrorsine *N*-oxide	9.0	1.4	2.3	0.5	2.9	0.8	5.8	1.1	9.0	0.7	1.1	69.2
usaramine	tr	0.0	1.2	0.4	1.1	0.3	0.6	0.1	0.6	0.0	tr	3.5
usaramine *N*-oxide	tr	0.0	3.3	0.7	13.5	4.5	0.2	0.1	11.0	1.0	tr	98.8
riddelliine	0.4	0.2	1.1	0.8	0.3	0.1	0.7	0.5	0.5	0.0	tr	4.3
riddelliine *N*-oxide	4.9	0.7	2.4	1.1	1.0	0.4	16.5	5.0	10.4	0.8	0.9	108.4
seneciphylline	55.4	13.6	12.8	2.7	101.4	45.2	24.3	6.6	37.1	1.7	1.2	102.0
seneciphylline *N*-oxide	673.0	151.6	181.1	46.4	627.6	203.1	361.8	86.9	517.1	27.4	12.4	1675.5
spartioidine	1.4	0.4	2.6	0.8	4.1	1.7	2.1	0.5	1.8	0.1	tr	6.0
spartioidine *N*-oxide	8.2	2.3	24.0	8.0	25.6	11.2	25.7	8.5	18.0	1.3	tr	68.1
acetylseneciphylline	11.1	6.5	15.9	4.4	11.1	3.4	4.4	1.0	8.9	0.4	1.9	31.4
acetylseneciphylline *N*-xide	145.0	76.3	158.9	52.6	103.5	37.3	108.8	30.4	149.7	8.4	29.6	465.9
senecivernine	0.6	0.1	0.4	0.1	0.7	0.2	0.3	0.1	0.4	0.0	tr	1.0
jacobine	5.6	3.8	881.9	179.7	168.7	43.8	137.5	29.3	58.4	4.0	0.9	367.4
jacobine *N*-oxide	10.7	1.7	571.8	97.5	246.4	44.9	884.3	172.0	217.6	11.8	2.4	934.1
jacoline	0.8	0.2	228.1	22.4	54.3	7.3	47.8	5.6	18.1	1.0	0.2	121.4
jacoline *N*-oxide	0.9	0.1	36.9	4.2	21.0	3.2	66.5	10.2	14.4	0.7	tr	63.9
jaconine	5.6	1.1	2253.4	297.8	576.6	105.8	448.6	103.0	204.5	14.6	2.4	1601.2
jaconine *N*-oxide	2.3	0.4	89.6	18.0	55.1	14.2	169.9	49.0	44.5	3.1	0.4	282.6
jacozine	0.9	0.2	18.7	3.8	5.8	1.2	2.9	0.6	3.0	0.2	tr	12.9
jacozine *N*-oxide	3.0	0.5	5.8	1.6	5.2	1.2	7.0	2.0	12.5	1.0	0.6	121.2
dehydrojaconine	7.8	1.8	149.9	15.5	61.5	11.0	27.5	5.8	33.7	1.9	1.0	167.5
erucifoline	7.1	1.7	18.7	2.7	5.6	1.4	19.4	4.6	14.7	0.6	3.3	59.5
erucifoline *N*-oxide	79.0	18.5	74.6	22.4	25.0	8.6	229.4	52.5	140.4	7.8	18.7	499.1
acetylerucifoline	2.5	0.5	10.4	3.1	2.2	1.0	6.6	1.7	4.1	0.2	tr	15.3
acetylerucifoline *N*-oxide	34.4	7.0	83.4	24.8	16.9	4.5	104.6	23.6	51.5	3.1	1.4	184.0
senkirkine	0.8	0.7	tr	0.0	0.1	0.0	tr	0.0	0.4	0.1	tr	4.7
otosenine	6.3	2.8	2.9	0.5	25.7	7.8	28.5	8.2	16.3	1.3	tr	177.6
onetine	1.3	0.6	0.5	0.0	5.5	1.3	4.6	0.7	3.4	0.2	tr	35.0
desacetyldoronine	3.6	1.6	2.2	0.3	28.2	7.8	20.4	3.5	16.4	1.5	tr	246.0
florosenine	6.5	2.0	0.0	0.0	8.1	2.3	4.6	1.0	3.6	0.3	0.0	34.6
floridanine	0.8	0.2	0.0	0.0	1.9	0.4	0.8	0.1	0.6	0.1	0.0	5.6
doronine	4.5	1.5	0.0	0.0	10.6	3.1	5.3	0.9	4.6	0.5	0.0	56.5
sum-fb	133.1	27.0	3604.7	259.6	1011.3	148.7	732.1	114.0	400.0	20.0	50.7	2348.5
sum-ox	1469.7	312.6	1331.7	278.2	130.1	344.8	2181.9	347.1	1435.9	59.3	267.4	3927.9
sum-sn	1442.4	321.2	513.0	137.6	1067.3	343.9	761.9	168.2	1018.5	50.1	86.6	2883.2
sum-jb	37.4	5.8	4236.2	316.2	1194.4	167.8	1792.0	250.6	606.6	30.5	14.8	3471.0
sum-er	123.0	26.1	187.2	50.3	49.7	14.6	360.0	80.6	210.7	10.9	34.8	662.2
sum-ot	24.4	5.8	6.1	0.4	80.7	18.8	64.7	9.8	45.8	3.3	0.6	558.5
Total	1627.2	337.8	4942.4	467.4	2392.2	470.7	2978.7	438.2	1892.1	71.2	334.0	6835.0

Means represent genotype mean concentrations, except in the case of the F_2 class, for which mean represents the mean concentration of F_2 genotype means. Max and Min of F_2 are the maximum and minimum genotype mean concentrations from among the F_2 lines. Sum-fb: the sum of all PA free bases. Sum-ox: the sum of all PA *N*-oxides. Sum-sn: the sum of all senecionine-like PAs. Sum-jb: the sum of all jacobine-like PAs. Sum-er: the sum of all erucifoline-like PAs. Sum-ot: the sum of all otosenine-like PAs, including florosenine, floridanine, and doronine. Total: sum of all PAs. Tr: trace amount, the concentration are less than 0.1µg g^{-1} DW.

- **Table S2.** PA concentrations (µg g^{-1} DW) in the roots of *J. aquatic* (one genotype), *J. vulgaris* (one genotype), F_1 hybrids (two genotypes) and F_2 hybrids (102 genotypes)

PAs	*J. aquatica*		*J. vulgaris*		F_1-A		F_1-B		F_2			
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Min	Max
senecionine	8.1	1.3	43.8	8.9	49.2	8.1	85.8	22.8	53.2	2.4	3.4	210.4
senecionine *N*-oxide	299.8	35.3	1303.6	187.7	1768.6	211.4	2201.1	288.0	1439.0	46.4	122.8	4564.9
integerrimine	2.1	0.3	14.1	2.6	6.4	1.0	10.3	3.0	6.9	0.3	1.1	26.4
integerrimine *N*-oxide	89.2	10.4	464.9	49.4	260.4	33.4	340.3	40.6	226.9	6.5	55.3	641.0
retrorsine	0.4	0.1	1.0	0.3	0.8	0.2	1.4	0.3	1.8	0.1	0.2	12.4
retrorsine *N*-oxide	12.2	0.9	20.2	3.0	14.4	2.9	28.2	2.8	38.1	1.6	8.7	267.5
usaramine	tr	0.0	0.4	0.1	0.6	0.1	0.2	0.0	0.6	0.0	tr	4.8
usaramine *N*-oxide	tr	0.0	12.0	1.6	23.2	5.2	0.4	0.2	16.5	1.2	tr	121.7
riddelliine	tr	0.0	0.3	0.1	0.2	0.1	0.3	0.1	0.5	0.0	tr	5.7
riddelliine *N*-oxide	6.8	1.2	19.6	2.2	2.2	0.5	17.8	3.0	15.4	0.6	2.6	80.4
seneciphylline	8.3	1.4	20.2	3.9	21.4	3.7	20.4	4.4	25.3	1.1	4.4	109.9
seneciphylline *N*-oxide	257.7	35.6	584.9	59.5	717.8	109.4	390.2	45.2	611.2	17.5	103.1	1572.2
spartioidine	tr	0.0	0.4	0.1	0.3	0.0	0.2	0.1	0.4	0.0	tr	1.7
spartioidine *N*-oxide	0.2	0.1	5.5	1.5	5.4	1.5	4.3	1.0	6.0	0.4	tr	24.7
acetylseneciphylline	22.1	3.3	74.0	10.9	47.4	9.8	35.2	7.9	47.2	1.7	13.8	142.0
acetylseneciphylline *N*-oxide	790.9	97.1	1620.1	182.0	1107.2	163.6	608.7	99.1	993.4	27.3	307.6	2239.3
senecivernine	0.3	0.0	1.8	0.4	0.5	0.1	0.9	0.2	0.8	0.0	0.2	2.2
jacobine	2.9	2.0	130.7	11.2	29.5	7.2	66.9	8.4	26.6	1.6	0.5	142.4
jacobine *N*-oxide	6.6	0.8	1470.1	140.5	312.9	68.7	591.7	105.6	221.0	11.5	3.2	718.4
jacoline	0.6	0.1	42.5	2.0	11.2	1.9	23.1	3.3	8.6	0.5	0.2	42.9
jacoline *N*-oxide	0.4	0.1	73.8	5.6	16.2	3.0	32.2	4.7	11.9	0.6	0.2	41.1
jaconine	2.8	0.8	107.2	18.8	34.7	7.0	96.6	27.3	30.1	2.0	0.8	123.4
jaconine *N*-oxide	1.2	0.3	91.6	12.2	36.6	8.8	67.5	22.3	22.6	1.4	0.5	102.3
jacozine	0.4	0.1	1.7	0.2	0.4	0.1	0.7	0.1	0.7	0.1	tr	6.8
jacozine *N*-oxide	1.7	0.2	12.5	1.5	3.8	0.6	3.2	0.5	8.9	0.4	0.6	49.3
dehydrojaconine	1.6	0.3	4.8	0.9	2.0	0.4	1.9	0.4	2.6	0.2	tr	20.5
erucifoline	0.9	0.2	3.8	0.7	0.8	0.2	2.7	0.3	3.2	0.1	0.7	13.7
erucifoline *N*-oxide	10.8	1.3	65.7	6.6	9.3	1.5	32.5	3.6	39.5	1.3	10.4	101.9
acetylerucifoline	0.2	0.0	0.2	0.1	tr	0.0	0.2	0.0	0.4	0.0	tr	1.8
acetylerucifoline *N*-oxide	1.7	0.2	6.6	1.7	1.1	0.2	3.4	0.9	9.0	0.4	1.6	39.1
senkirkine	0.2	0.1	8.2	1.3	15.2	3.7	11.8	1.7	17.4	1.7	tr	255.0
otosenine	0.8	0.2	263.1	24.7	66.3	16.3	84.8	14.6	68.9	3.3	tr	366.1
onetine	0.2	0.1	32.3	1.7	11.2	2.7	13.0	1.2	11.9	0.5	tr	59.5
desacetyldoronine	0.4	0.1	54.9	8.0	22.2	6.7	30.8	8.1	21.9	1.4	tr	119.5
florosenine	tr	0.0	0.2	0.1	14.4	3.6	9.8	1.9	4.7	0.4	0.0	32.9
floridanine	0.0	0.0	0.0	0.0	1.8	0.4	1.3	0.2	0.7	0.1	0.0	5.1
doronine	tr	0.0	0.0	0.0	5.9	2.1	4.9	1.3	2.3	0.2	0.0	17.4
sum-fb	50.8	8.2	445.0	41.6	205.2	29.4	346.0	63.4	208.0	6.9	50.6	542.1
sum-ox	1479.2	172.0	5751.3	496.9	4279.0	566.7	4321.5	501.6	3659.4	90.6	909.9	8649.0
sum-sn	1498.2	176.8	4185.0	465.8	4025.5	533.6	3744.8	470.4	3482.4	88.6	842.2	8083.5
sum-jb	18.2	2.6	1935.1	136.7	447.3	88.4	883.8	132.5	332.9	15.7	14.7	1028.0
sum-er	13.6	1.4	76.3	8.4	11.3	1.5	38.9	4.4	52.1	1.6	14.1	134.1
sum-ot	1.6	0.3	350.6	21.2	121.8	29.3	144.8	19.5	110.3	4.9	0.4	592.4
Total	1532.2	179.4	6557.0	499.5	4621.8	613.2	4825.0	537.2	3996.0	97.2	984.8	9421.1

Means represent genotype mean concentrations, except in the case of the F_2 class, for which mean represents the mean concentration of F_2 genotype means. Max and Min of F_2 are the maximum and minimum genotype mean concentrations from among the F_2 lines. Sum-fb: the sum of all PA free bases. Sum-ox: the sum of all PA *N*-oxides. Sum-sn: the sum of all senecionine-like PAs. Sum-jb: the sum of all jacobine-like PAs. Sum-er: the sum of all erucifoline-like PAs. Sum-ot: the sum of all otosenine-like PAs, including florosenine, floridanine, and doronine. Total: sum of all PAs. Tr: trace amount, the concentration are less than 0.1µg g^{-1} DW.

- **Table S3**: Quantitative variation of PAs in the shoots and roots of two F_1 and 102 F_2 hybrids relative to parental genotypes (one genotype each of *J. aquatica* and *J. vulgaris*)

Group	PAs	Codes	Shoots								Roots							
			F_1-A	F_1-B	F_2						F_1-A	F_1-B	F_2					
					U[d]	Dl	Im	Dh	O	ND			U	Dl	Im	Dh	O	ND
Senecionine-like PAs (simple senecionine-related derivatives)	senecionine	sn[b]	Im	Im	1[e]	55	18	28	0	0	Dh	Dh	0	28	0	0	0	74
	senecionine *N*-oxide	snox[b]	Im	Im	2	52	13	35	0	0	Dh	Dh	0	40	0	0	0	62
	integerrimine	ir[b]	ND	ND	15	27	2	8	0	50	ND	ND	0	11	0	3	0	88
	integerrimine *N*-oxide	irox	ND	ND	6	29	0	14	1	52	Dh	Dh	0	34	42	26	0	0
	retrorsine	rt	Dl	Dl	0	58	0	14	0	30	ND	ND	0	1	0	26	32	43
	retrorsine *N*-oxide	rtox	Dl	Dh	0	36	0	46	6	14	ND	ND	0	3	0	16	52	31
	usaramine	rd	Dh	Dh	0	56	0	33	6	7	Dh	ND	0	17	0	0	0	85
	usaramine *N*-oxide	rdox[b]	Dh	ND	0	0	73	29	0	0	Dh	ND	0	25	0	0	0	77
	riddelliine	us[b]	ND	ND	0	4	0	3	4	91	ND	ND	0	0	0	5	12	85
	riddelliine *N*-oxide	usox[a,b]	Dl	Dh	0	4	0	34	11	53	Dl	ND	0	9	0	3	0	90
	seneciphylline	sp	Dh	Dl	3	35	0	51	0	13	ND	ND	0	8	0	38	16	40
	seneciphylline *N*-oxide	spox	Dh	ND	2	21	0	45	0	34	Dh	ND	3	20	0	57	9	13
	spartioidine	st[a]	ND	ND	0	4	98	0	0	0	ND	ND	0	13	0	33	10	46
	spartioidine *N*-oxide	stox[a,b]	ND	ND	0	3	96	0	3	0	ND	ND	0	25	0	0	0	77
	acetylseneciphylline	acsp[b]	ND	Dl	0	37	0	14	0	51	ND	ND	0	2	0	3	0	97
	acetylseneciphylline *N*-oxide	acspox[b]	ND	ND	0	1	0	2	2	97	ND	Dl	0	0	0	17	0	85
	senecivernine	sv	ND	Dh	0	0	0	39	0	63	ND	ND	0	68	1	32	0	1
Jacobine-like PAs (jacobine-related derivatives)	jacobine	jb	Im	Im	0	29	73	0	0	0	Dh	Dh	0	32	62	8	0	0
	jacobine *N*-oxide	jbox	Im	Dh	7	23	39	33	0	0	Dh	Dh	0	23	76	3	0	0
	jacoline	jl	Im	Im	0	24	78	0	0	0	Dh	Dh	0	28	70	4	0	0
	jacoline *N*-oxide	jlox	Im	O	0	32	44	25	1	0	Dh	Dh	0	28	74	0	0	0
	jaconine	jn	Im	Im	0	21	78	3	0	0	Dh	Dh	0	44	26	32	0	0
	jaconine *N*-oxide	jnox	Dh	Dh	0	33	26	41	2	0	Dh	Dh	0	37	45	20	0	0
	jacozine	jz	Im	Im	0	53	47	2	0	0	Dl	ND	3	78	1	18	0	2
	jacozine *N*-oxide	jzox	ND	ND	0	15	0	14	27	46	Dl	Dl	0	28	26	41	7	0
	dehydrojaconine	dhjn	Im	Im	4	36	52	10	0	0	ND	ND	8	51	0	13	2	28
Erucifoline-like PAs (erucifoline-related derivatives)	erucifoline	er	Dl	Dh	0	42	0	35	5	20	Dl	ND	0	25	0	58	7	12
	erucifoline *N*-oxide	erox	Dl	Dh	2	5	0	20	13	62	Dl	ND	1	21	38	42	0	0
	acetylerucifoline	acer	Dl	Dh	2	65	0	23	0	12	ND	ND	0	0	0	5	17	80
	acetylerucifoline *N*-oxide	acerox	Dl	ND	10	19	0	4	1	68	ND	ND	0	4	0	42	28	28
Otosenine-like PAs (otosenine-related derivatives)	senkirkine	sk[a, b]	ND	ND	0	3	96	0	3	0	Dh	Dh	0	36	0	0	2	64
	otosenine	ot[b]	O	O	14	3	0	2	40	43	Im	Dh	0	44	0	46	0	12
	onetine	one[a]	Dh	Dh	0	0	83	14	5	0	Dh	Dh	0	13	73	15	1	0
	desacetyldoronine	desdor	O	O	10	2	0	2	44	44	Dh	Dh	0	15	53	34	0	0
Sum					77	772	898	595	174	850			15	783	587	640	195	1146
Percentage (%)					2.3	22.9	26.7	17.7	5.2	25.3			0.4	23.3	17.4	19.0	5.8	34.0
Totals	Sum of PA free bases	sum-fb	Im	Im	4	39	58	1	0	0	Dh	Dh	0	16	51	35	0	0
	Sum of PA *N*-oxides	sum-ox	ND	ND	13	4	0	3	1	81	Dh	Dh	0	26	31	45	0	0
	sum of all senecionine-like PAs	sum-sn	Dl	ND	1	34	0	25	0	42	Dh	Dh	0	30	0	64	3	5
	sum of all jacobine-like PAs	sum-jb	Im	Im	3	17	80	2	0	0	Dh	Dh	0	18	82	2	0	0
	sum of all erucifoline-like PAs	sum-er	U	ND	6	2	0	4	11	79	Dl	ND	1	16	32	52	1	0
	sum of all otosenine-likePAs[c]	sum-ot[a,b]	O	O	0	0	86	15	1	0	Dh	Dh	0	43	0	47	0	12
	Total PA		Dl	Dh	14	64	0	7	0	17	Dh	Dh	0	18	46	38	0	0
Sum					41	160	224	57	13	219			1	167	242	283	4	17
Percentage (%)					5.7	22.4	31.4	8.0	1.8	30.7			0.1	23.4	33.9	39.6	0.6	2.4

[a,b] the variables were not normally distributed and were analyzed using non parametric methods for shoot and root samples separately, a = shoots, b = roots.
[c] including florosenine, floridanine, and doronine
[d] U (under-expression, significantly less than that of both parents); Dl (dominant to the parent with lower expression, not different from the parent with lower expression and significantly different from the other parent); intermediate to the parents (Im, intermediate to but significantly different from both parents); Dh (dominant to the parent with higher expression, not different from the parent with higher expression and significantly different from the other parent); O (over-expression, significantly greater than that of both parents); ND (not significantly different from the parents).
[e] Numbers indicate the number of F_2 genotypes in which a particular PAs shown particular type of variation.

- **Table S4** Coefficients (r_s) of Spearman Rank correlation between the individual PA in the shoots (above the diagonal line) and roots (below diagonal line) of *J. aquatic* (one genotype), *J. vulgaris* (one genotype), F_1 hybrids (two genotypes) and F_2 hybrids (102 genotypes)

	Senecionine-like PAs																	Jacobine-like PAs										Erucifoline-like PAs				Otosenine-like PAs							
	sn	snox	ir	irox	rt	rtox	us	usox	rd	rdox	sp	spox	st	stox	acsp	acspox	sv	jb	jbox	jl	jlox	jn	jnox	jz	jzox	dhjn	er	erox	acer	acerox	sk	ot	one	desdor	fs	fd	dor	
sn		0.89	0.83	0.73	0.33	0.55	0.11	-0.03	0.07	0.08	0.70	0.62	0.23	0.19	0.55	0.49	0.62	-0.19	-0.03	-0.12	-0.04	0.01	0.11	-0.20	-0.02	0.02	0.19	0.25	0.19	0.21	0.14	0.13	0.22	0.30	0.01	0.07	0.11	sn
snox	0.68		0.78	0.89	0.23	0.62	0.07	0.00	0.13	0.21	0.68	0.76	0.28	0.32	0.53	0.66	0.57	-0.09	0.08	-0.06	0.04	0.03	0.18	-0.10	0.20	0.11	0.26	0.44	0.27	0.39	0.09	0.20	0.27	0.33	0.07	0.12	0.16	snox
ir	0.93	0.53		0.84	0.38	0.53	0.13	0.01	0.18	0.10	0.75	0.68	0.42	0.39	0.67	0.61	0.74	-0.01	0.18	0.10	0.17	0.24	0.36	-0.12	0.03	0.20	0.32	0.38	0.38	0.38	0.11	0.09	0.22	0.32	-0.03	0.05	0.11	ir
irox	0.64	0.92	0.59		0.25	0.61	0.07	0.03	0.20	0.27	0.71	0.83	0.40	0.49	0.59	0.76	0.63	0.08	0.28	0.13	0.25	0.22	0.42	0.00	0.27	0.25	0.33	0.57	0.41	0.55	0.04	0.16	0.24	0.32	0.04	0.10	0.15	irox
rt	0.53	0.15	0.52	0.13		0.39	0.35	0.21	0.16	0.15	0.22	0.14	0.11	0.05	0.34	0.20	0.31	0.02	0.12	0.13	0.14	0.28	0.26	-0.12	-0.18	0.12	0.18	0.07	0.07	-0.01	0.20	0.00	0.10	0.20	0.04	0.09	0.16	rt
rtox	0.22	0.23	0.18	0.21	0.69		0.15	0.23	0.30	0.40	0.43	0.53	0.11	0.20	0.35	0.48	0.37	-0.14	0.05	-0.16	0.00	-0.08	0.12	-0.22	0.18	-0.09	0.20	0.46	0.19	0.36	0.03	0.08	0.10	0.15	0.04	0.06	0.10	rtox
us	0.31	0.15	0.29	0.12	0.35	0.24		0.56	0.06	0.20	0.11	0.07	0.06	0.02	0.12	0.06	0.16	0.21	0.15	0.27	0.18	0.36	0.22	0.16	-0.06	0.32	0.08	0.02	0.08	0.03	0.12	0.04	0.10	0.16	0.17	0.19	0.22	us
usox	0.12	0.11	0.10	0.09	0.23	0.29	0.63		0.21	0.15	0.01	0.04	-0.09	-0.03	0.09	0.08	-0.01	0.14	-0.01	0.13	0.01	0.18	0.03	0.20	0.02	0.28	0.09	0.06	0.06	0.04	0.03	0.07	0.09	0.13	0.23	0.22	0.25	usox
rd	0.23	0.05	0.25	0.06	0.43	0.34	0.25	0.22		0.19	0.19	0.23	0.18	0.25	0.20	0.27	0.18	-0.13	-0.07	-0.11	-0.08	-0.03	0.03	-0.08	0.13	0.08	0.18	0.25	0.17	0.18	0.03	-0.05	-0.01	0.07	0.08	0.07	0.11	rd
rdox	-0.08	0.10	-0.07	0.16	0.13	0.30	0.28	0.23	0.27		0.20	0.35	0.21	0.26	0.03	0.20	0.21	0.05	0.17	0.01	0.13	-0.01	0.15	0.06	0.46	0.05	0.31	0.54	0.23	0.41	0.14	0.01	-0.02	-0.04	0.02	0.00	0.00	rdox
sp	0.82	0.47	0.83	0.49	0.50	0.17	0.34	0.21	0.31	-0.01		0.90	0.66	0.61	0.56	0.60	0.72	-0.02	0.12	0.03	0.08	0.18	0.29	0.08	0.42	0.37	0.38	0.49	0.38	0.43	0.06	-0.01	0.10	0.21	-0.01	0.06	0.10	sp
spox	0.54	0.75	0.50	0.78	0.10	0.14	0.21	0.22	0.13	0.19	0.68		0.60	0.65	0.50	0.70	0.62	0.04	0.20	0.04	0.14	0.14	0.32	0.13	0.59	0.35	0.36	0.65	0.39	0.58	0.01	0.04	0.10	0.18	0.03	0.07	0.10	spox
st	0.43	0.17	0.47	0.19	0.23	-0.06	0.16	0.04	0.21	0.05	0.60	0.38		0.87	0.38	0.43	0.55	0.13	0.26	0.21	0.24	0.33	0.40	0.21	0.55	0.49	0.52	0.54	0.46	0.45	0.07	-0.09	0.00	0.13	-0.07	-0.03	0.04	st
stox	0.24	0.17	0.26	0.20	-0.02	-0.15	-0.06	-0.02	0.08	0.00	0.39	0.40	0.59		0.34	0.51	0.50	0.15	0.29	0.21	0.26	0.32	0.43	0.19	0.63	0.49	0.52	0.66	0.51	0.57	0.00	-0.10	-0.03	0.10	-0.06	-0.04	0.05	stox
acsp	0.74	0.35	0.83	0.44	0.42	0.13	0.30	0.18	0.26	-0.01	0.80	0.49	0.54	0.40		0.78	0.59	-0.03	0.05	0.07	0.06	0.21	0.22	-0.02	0.03	0.27	0.28	0.25	0.31	0.25	0.23	0.11	0.23	0.34	0.03	0.10	0.15	acsp
acspox	0.38	0.60	0.40	0.72	-0.05	0.07	0.07	0.13	0.04	0.15	0.39	0.70	0.27	0.37	0.58		0.54	-0.02	0.11	0.04	0.08	0.17	0.28	-0.01	0.30	0.30	0.35	0.52	0.40	0.49	0.11	0.11	0.20	0.30	0.11	0.16	0.22	acspox
sv	0.80	0.38	0.85	0.42	0.53	0.18	0.37	0.15	0.30	0.07	0.76	0.40	0.49	0.28	0.76	0.29		-0.01	0.15	0.10	0.15	0.25	0.33	-0.04	0.19	0.28	0.44	0.44	0.43	0.40	0.16	-0.01	0.12	0.23	-0.06	0.01	0.06	sv
jb	0.11	0.27	0.13	0.38	0.12	0.11	0.17	0.10	0.06	0.20	0.12	0.27	0.11	0.15	0.08	0.22	0.12		0.87	0.92	0.87	0.77	0.73	0.65	0.18	0.50	0.00	0.01	0.11	0.16	0.13	0.35	0.29	0.21	0.11	0.11	0.07	jb
jbox	-0.01	0.26	0.02	0.39	-0.02	0.08	0.09	0.07	-0.04	0.19	-0.05	0.22	0.01	0.09	-0.06	0.21	-0.01	0.88		0.86	0.98	0.77	0.89	0.44	0.22	0.38	0.04	0.17	0.16	0.28	0.14	0.28	0.26	0.22	0.00	0.01	0.01	jbox
jl	0.12	0.25	0.15	0.37	0.14	0.12	0.21	0.13	0.08	0.20	0.13	0.26	0.13	0.17	0.08	0.18	0.14	0.92	0.89		0.90	0.91	0.83	0.59	0.07	0.61	0.07	-0.01	0.17	0.15	0.14	0.28	0.31	0.28	0.07	0.10	0.09	jl
jlox	0.02	0.23	0.06	0.36	0.01	0.08	0.13	0.09	-0.02	0.20	-0.02	0.20	0.04	0.08	-0.03	0.18	0.03	0.87	0.98	0.91		0.81	0.90	0.44	0.14	0.41	0.04	0.11	0.15	0.22	0.14	0.27	0.28	0.24	0.01	0.02	0.02	jlox
jn	0.39	0.21	0.46	0.33	0.29	0.04	0.31	0.12	0.12	0.03	0.38	0.23	0.32	0.25	0.37	0.17	0.40	0.75	0.70	0.81	0.74		0.88	0.45	0.00	0.70	0.18	0.05	0.28	0.18	0.17	0.16	0.26	0.35	0.07	0.11	0.17	jn
jnox	0.22	0.25	0.30	0.38	0.11	0.04	0.20	0.10	0.03	0.09	0.18	0.24	0.19	0.21	0.22	0.25	0.22	0.80	0.86	0.85	0.88	0.91		0.29	0.14	0.51	0.18	0.25	0.32	0.35	0.13	0.14	0.22	0.30	0.00	0.03	0.08	jnox
jz	0.02	0.19	0.01	0.24	0.06	0.05	0.20	0.15	0.12	0.32	0.21	0.37	0.21	0.18	0.04	0.16	0.10	0.47	0.33	0.44	0.33	0.26	0.20		0.43	0.70	0.14	0.07	0.11	0.14	0.05	0.34	0.28	0.19	0.19	0.20	0.13	jz
jzox	-0.07	0.23	-0.10	0.26	-0.18	-0.01	0.03	0.15	0.10	0.40	0.17	0.54	0.33	0.49	0.11	0.46	-0.02	0.20	0.16	0.17	0.12	-0.07	0.02	0.46		0.34	0.36	0.62	0.27	0.49	-0.02	0.04	-0.04	-0.08	-0.01	-0.04	-0.07	jzox
dhjn	0.46	0.26	0.52	0.34	0.31	0.05	0.32	0.21	0.22	0.06	0.65	0.47	0.53	0.43	0.54	0.30	0.48	0.41	0.26	0.44	0.29	0.67	0.52	0.46	0.23		0.40	0.22	0.38	0.27	0.08	0.15	0.25	0.34	0.18	0.23	0.25	dhjn
er	0.55	0.22	0.59	0.25	0.45	0.12	0.26	0.17	0.33	0.21	0.66	0.36	0.51	0.37	0.52	0.13	0.61	0.10	-0.04	0.11	0.00	0.33	0.13	0.22	0.18	0.52		0.77	0.69	0.59	0.03	0.02	0.09	0.20	-0.03	-0.01	0.05	er
erox	0.09	0.24	0.13	0.32	0.02	0.11	0.07	0.14	0.16	0.53	0.19	0.39	0.25	0.30	0.17	0.34	0.16	0.15	0.18	0.13	0.17	0.07	0.14	0.24	0.51	0.21	0.56		0.62	0.78	-0.02	0.00	0.01	0.09	-0.02	-0.04	0.03	erox
acer	0.33	0.02	0.40	0.03	0.33	0.10	0.24	0.12	0.19	0.19	0.40	0.11	0.34	0.15	0.40	0.08	0.43	-0.09	-0.21	-0.07	-0.16	0.12	-0.03	0.06	0.03	0.25	0.47	0.20		0.86	-0.09	-0.07	0.00	0.11	-0.10	-0.09	-0.02	acer
acerox	0.02	0.14	0.02	0.16	0.04	0.19	0.00	0.10	0.13	0.41	0.09	0.25	0.16	0.23	0.15	0.37	0.08	0.00	-0.04	-0.03	-0.07	-0.16	-0.10	0.13	0.49	0.00	0.28	0.53	0.41		-0.09	0.00	0.01	0.06	-0.05	-0.07	-0.02	acerox
sk	0.39	0.42	0.33	0.42	0.15	0.11	0.06	0.07	-0.01	-0.02	0.24	0.25	0.06	0.05	0.19	0.20	0.26	0.19	0.22	0.16	0.21	0.16	0.20	0.01	-0.02	0.08	0.19	0.16	-0.02	-0.03		0.31	0.33	0.34	0.16	0.16	0.19	sk
ot	0.20	0.41	0.16	0.47	0.10	0.15	0.10	0.18	0.05	0.20	0.16	0.34	0.05	0.08	0.08	0.28	0.14	0.46	0.47	0.45	0.45	0.25	0.31	0.35	0.25	0.18	0.15	0.27	-0.09	0.09	0.66		0.93	0.83	0.52	0.53	0.47	ot
one	0.33	0.40	0.31	0.47	0.22	0.16	0.18	0.22	0.11	0.17	0.29	0.35	0.15	0.11	0.22	0.27	0.29	0.45	0.43	0.47	0.44	0.36	0.37	0.34	0.17	0.31	0.27	0.26	0.01	0.05	0.66	0.95		0.91	0.47	0.53	0.49	one
desdor	0.55	0.37	0.58	0.45	0.31	0.10	0.25	0.19	0.12	0.01	0.49	0.34	0.30	0.22	0.47	0.28	0.48	0.32	0.29	0.37	0.33	0.55	0.49	0.12	-0.03	0.52	0.40	0.20	0.16	-0.07	0.62	0.69	0.81		0.51	0.56	0.59	desdor
fs	-0.01	0.05	-0.01	0.07	0.05	0.04	0.12	0.14	0.06	0.07	0.05	0.07	0.02	0.03	0.01	0.02	-0.03	0.13	0.15	0.14	0.16	0.15	0.17	0.09	-0.04	0.12	0.06	0.13	-0.01	-0.04	0.27	0.14	0.16	0.23		0.86	0.86	fs
fd	0.08	0.09	0.08	0.12	0.10	0.09	0.16	0.18	0.09	0.09	0.12	0.10	0.08	0.06	0.07	0.04	0.05	0.11	0.14	0.13	0.15	0.18	0.19	0.06	-0.07	0.16	0.13	0.17	0.02	-0.04	0.32	0.16	0.21	0.31	0.90		0.87	fd
dor	0.16	0.08	0.16	0.09	0.13	0.05	0.19	0.18	0.08	0.04	0.18	0.09	0.13	0.09	0.16	0.03	0.12	0.08	0.09	0.11	0.11	0.24	0.22	0.01	-0.12	0.23	0.18	0.14	0.09	-0.08	0.26	0.06	0.14	0.34	0.84	0.86		dor
	sn	snox	ir	irox	rt	rtox	us	usox	rd	rdox	sp	spox	st	stox	acsp	acspox	sv	jb	jbox	jl	jlox	jn	jnox	jz	jzox	dhjn	er	erox	acer	acerox	sk	ot	one	desdor	fs	fd	dor	

Correlation testes were contacted by using the absolute concentrations (µg g^{-1} DW) data. In all cases: $df = 607$.
The P values were adjusted using sequential Bonferroni methods and were indicated by the background color of the cells:
white: $P > 0.01$; yellow: P: 0.001-0.01; orange: $P < 0.001$. Abbreviations for PAs are defined in Table 1.

Chapter 3

The genotype-dependent presence of pyrrolizidine alkaloids as tertiary amine in *Jacobaea vulgaris*

Lotte Joosten, Dandan Cheng, Patrick P.J. Mulder, Klaas Vrieling, Johannes A. van Veen, Peter G.L. Klinkhamer

Lotte Joosten and Dandan Cheng contributed equally to this work.

Secondary metabolites such as pyrrolizidine alkaloids (PAs) play a crucial part in plant defense. PAs can occur in plants in two forms: tertiary amine (free base) and *N*-oxide. PA extraction and detection are of great importance for the understanding of the role of PAs as plant defense compounds, as the tertiary PA form is known for its stronger influence on several generalist insects, whereas the *N*-oxide form is claimed to be less deterrent. We measured PA *N*-oxides and their reduced tertiary amines by liquid chromatography–tandem mass spectrometry (LC-MS/MS). We show that the occurrence of tertiary PAs is not an artifact of the extraction and detection method. We found up to 50% of tertiary PAs in shoots of Jacobine – chemotype plants of *Jacobaea vulgaris*. Jacobine and its derivatives (jacoline, jaconine, jacozine and dehydrojaconine) may occur for more than 20% in reduced form in the shoots and more than 10% in the roots. For 22 PAs detected in F_2 hybrids (*J. vulgaris* × *Jacobaea aquatica*), we calculated the tertiary amine percentage (TA% = the tertiary amine concentration/(tertiary amine concentration + the corresponding *N*-oxide concentration) × 100). We found that the TA% for various PAs was genotype-dependent. Furthermore, TA% for the different PAs were correlated and the highest correlations occurred between PAs which share high structural similarity.

Keywords: *Senecio, Jacobaea vulgaris; Jacobaea aquatica*; *Asteraceae*; Quantitative descriptive analysis; Pyrrolizidine alkaloid; *N*-oxides; Hybrids; Plant defense; Secondary metabolite diversity

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1. Introduction

Pyrrolizidine alkaloids (PAs) are a well known class of defense compounds with a wide variety of structures. From several genera of *Asteraceae*, *Boraginaceae*, *Orchidaceae* and *Fabaceae*, more than 360 structurally different PAs have been isolated (Rizk, 1991; Hartmann and Witte 1995). It is known that PAs are present as mixtures of the tertiary alkaloids and the respective *N*-oxides in plants (Rizk, 1991). It is generally accepted that in *Senecio* and *Jacobaea* plants PAs occur mainly or even exclusively in *N*-oxide form (Hartmann and Toppel, 1987; Hartmann et al, 2004; Cao et al, 2008; Kempf et al, 2010).

In several *Senecio* and *Jacobaea* species, such as *Senecio vulgaris*, PAs are synthesized in the roots primarily as senecionine *N*-oxide (Hartmann and Toppel, 1987; Toppel et al, 1987). Subsequently, senecionine *N*-oxide is transported to the shoot, where by specific enzymes, further diversification into different individual PAs takes place (Hartmann and Dierich, 1998). The water soluble *N*-oxide form is considered to be ideal for phloem transport (Hartmann et al, 1989) and storage in cell vacuoles (von Borstel and Hartmann, 1986; Ehmke et al, 1988).

Generalist insect herbivores reduce *N*-oxides in the gut to tertiary PAs, where these tertiary PAs are passively taken up into the body and when converted into pyrroles they are toxic by acting as highly reactive alkylating agents in mammals and fruit flies (Mattocks, 1986; Frei et al, 1992). Since the PA *N*-oxides are reduced in the herbivore's gut, we could expect that it displays the same degree of toxicity as the respective tertiary amines, however in several studies it was shown that individual PA *N*-oxides showed less deterrent or toxic effects for some generalist insect herbivores compared to the tertiary PAs (Dreyer et al, 1985; van Dam et al, 1995; Macel et al, 2005). van Dam et al (1995) found that three PAs from *Cynoglossum officinale* equally deterred feeding by *Spodoptera exigua* larvae, but the tertiary PA form deterred feeding more efficiently than the corresponding PA *N*-oxides. Macel et al (2005) showed that retrorsine *N*-oxide was significantly less repellent to the locust *Locusta migratoria* compared to the corresponding tertiary PA. After 6 days on a diet of retrorsine *N*-oxide 60% of the thrips *Frankliniella occidentalis* survived against 0% on the tertiary PA. Specialist insects, i.e, some butterflies and moths (Lepidoptera), certain chrysomelid leaf beetles (Coleoptera) and the grasshopper *Zonocerus variegates* are adapted to PAs, sequestrate the tertiary PAs and specifically convert them into *N*-oxides which they store and utilize for their own chemical defense (Boppre 1986; Lindigkeit et al, 1997; Dobler 2001; Nishida 2002; Narberhaus et al, 2003).

For many years, PAs were typically isolated by acid-base extraction in combination with zinc reduction. Gas chromatography (GC) with flame ionisation detection (FID), nitrogen phosphorus detection (NPD) or mass spectrometric detection (MS) have typically been used as analytical methods. Recently liquid chromatography–tandem mass spectrometry (LC-MS/MS) has been introduced for measuring PAs in plant material. Unlike GC-related methods, LC-MS/MS and NMR can detect both tertiary amines and *N*-oxides without an additional reducing step (Crews et al, 2010; Joosten et al, 2010). However, NMR needs relatively high concentrations of PAs for detection. LC-MS/MS is therefore a suitable and sensitive method to detect both forms of PAs.

We used LC-MS/MS to detect both forms of PAs. We found consistently large amounts of tertiary amines in *Jacobaea vulgaris* plants (Joosten et al, 2009, 2010). However, the general tendency in literature is that tertiary amines are present only in very small amounts and maybe are due to artifacts during extraction or detection (Hartmann and Toppel, 1987; Hartmann, 1999; Hartmann and Ober, 2000). PA *N*-oxides from *Senecio* plants are relatively unstable and are easily converted into their reduced form, the pre-toxic tertiary PAs under various experimental conditions. For example, the reduction increased upon prolonged heating of the sample (e.g. soxhlet extraction), when the amino acid cysteine was added and in the presence of plant material (Hartmann and Toppel, 1987; Hösch et al, 1996). Therefore we tested our method for possible artifacts by several PA reduction and oxidation experiments with chemical agents and plant material.

Further proof of the presence of tertiary amines in living plant tissue can be obtained by showing that the concentrations of tertiary amines have a genetic basis and result from transformations by specific enzymes. It is already known that variation in composition and concentration of PAs in *J. vulgaris* has a large genetic component (Vrieling et al, 1993; Macel et al, 2004). In order to assess the genetic basis in the variation, the occurrence of the tertiary amine form, we conducted a crossing of *J. vulgaris*, which has high levels of tertiary amines, with the closely related *Jacobaea aquatica* (syn. *Senecio aquaticus*), which has low levels of tertiary amines (Cheng et al, manuscript in preparation).

Here we report on studies to obtain a better understanding of the (bio)chemistry of PAs in above and below ground plant parts of *J. vulgaris* and hence on the mechanisms of their activity as defense compounds against herbivores. Thus, we investigated: (1) the chemical reduction of three different PA *N*-oxides (representatives of the three structural groups) to assess the chemical PA (in)stability towards two different reducing agents; (2) the chemical oxidation of three different tertiary PAs to assess the chemical PA (in)stability towards an oxidation agent; (3) the spontaneous reduction of three different PA *N*-oxides in the presence of possibly reducing agents as well as the spontaneous *N*-oxidation of three different tertiary PA in the presence of possibly oxidation agents naturally occurring in plant material of several different *Asteraceae* species; (4) the spontaneous reduction of PAs during freeze-drying compared to immediate PA extraction from freshly ground material under liquid nitrogen; (5) the PA distribution in five different *J. vulgaris* genotypes by using an LC-MS/MS method for simultaneous measurement of PA *N*-oxides and tertiary PAs and (6) the genotype effect on the tertiary alkaloid relative content (TA%) for different PAs in the hybrids and the correlation between the TA% of different PAs.

2. Material and Methods

2.1. Standard PA extraction for LC-MS/MS

Freeze-dried plant material (approximately 10 mg) was extracted in 1 ml 2% formic acid. Heliotrine was added as internal standard to the extraction solvent at a concentration of 1 µg/ml. The plant extract solution was shaken for 30 min. After centrifugation the residual plant material was removed by filtering the extraction solution through a 0.2 µm nylon membrane (Acrodisk® 13 mm syringe filter). An aliquot of 25 µl filtered solution was diluted with 975 µl water and 10 µl was injected in the LC-MS/MS system.

2.2. Standard PA analysis by LC-MS/MS

A Waters Acquity ultra performance liquid chromatographic (UPLC) system coupled to a Waters Quattro Premier XE tandem mass spectrometer (Waters, Milford, PA, USA) was used for PA determination. Chromatographic separation was achieved on a Waters Acquity BEH C18 150 x 2.1 mm, 1.7 µm, UPLC column, kept at 50 °C and ran with a water/acetonitrile linear gradient containing 6.5 mM ammonia at a flow of 0.4 ml/min. The gradient started at 100% water and during analysis the acetonitrile percentage was raised to 50% in 12 min.

The MS system was operated in positive electrospray mode and data were recorded in multiple monitoring mode using two selected precursor ion to product ion transitions per compound. Cone

and collision energy settings were optimized for the individual compounds. Obtained peak areas were internally calibrated using the internal standard and the individual compounds were quantified against a standard solution of the PAs in an extract of the non-PA containing asterid *Tanacetum vulgare* to mimic the plant matrix. Seventeen individual PA standards were available for this study, representing over 90% of the total amount of PAs present in the plants extracts. Senecionine, seneciphylline, retrorsine and their *N*-oxides as well as senkirkine were available from commercial sources (Phytolab, Vestenbergsgreuth, Germany; Phytoplan, Heidelberg, Germany). Integerrimine was obtained as a kind gift of Dr. Trigo (UNICAMP, Campinas, Brazil). Riddelliine and its *N*-oxide were obtained as a kind gift from Dr. Chou (NCTR, Jefferson, AR, USA). Acetylseneciphylline was obtained by acetylation of seneciphylline with acetic anhydride and pyridine. Jacobine and erucifoline were isolated from *J. vulgaris* plant material (PRISNA, Leiden, The Netherlands). The identity of the standards isolated was confirmed by 1H-NMR and LC-MS analysis. *N*-oxides of integerrimine, jacobine, erucifoline and acetylseneciphylline were prepared by *N*-oxidation according to the method of Christie et al (1949), adapted by Chou et al (2003). The remaining PAs, being tertiary PAs as well as *N*-oxides, were quantified by using the response of a structurally related standard. Data processing was conducted with Masslynx 4.1 software.

2.3. Chemical reduction of PA *N*-oxides

A mixture of three PA *N*-oxides (senecionine *N*-oxide, jacobine *N*-oxide and erucifoline *N*-oxide, 1 µg /ml) was exposed to the reducing agent sodium metabisulfite ($Na_2S_2O_5$) in a range of 5 concentrations (0, 0.01, 0.03, 0.1, 0.3, 1 mM) in 2% formic acid solution. After 1, 4 and 24 h of incubation at room temperature the solutions were diluted 10-fold with water and injected in the LC-MS/MS system. The same mixture of standards was also exposed to the amino acid cysteine at three concentrations (1, 10 and 1000 mM), in two different solutions, 2% formic acid and water.

The relative amount of tertiary PA present in a sample was calculated as the measured concentration of tertiary PA divided by the sum of the concentration of tertiary PA and corresponding PA *N*-oxide.

A three-way ANOVA with two replications was used to analyze if PA type (senecionine *N*-oxide, jacobine *N*-oxide and erucifoline *N*-oxide), reducing agent concentration (0, 0.01, 0.03, 0.1, 0.3, 1 mM) and incubation time (1, 4, 24 h) have a significant influence on the relative concentration of tertiary PAs formed by reduction of the added PA *N*-oxides. The analysis was made by General Linear Model (GLM) univariate analyses procedure with the relative concentration of tertiary PAs as the dependent variable and PA type, reducing agent concentration and incubation time as fixed factors. All tests were conducted with SPSS 17.0 for Windows.

2.4. Chemical *N*-oxidation of tertiary PAs

The three individual tertiary PAs (senecionine, jacobine and erucifoline), were added to five concentrations (0.01, 0.03, 0.1, 0.3, 1 mM) of the oxidation agent hydrogen peroxide (HOOH) in 2% formic acid solution. After 1, 4 and 24 h of incubation at room temperature the solutions were diluted 10-fold with water and injected in the LC-MS/MS system.

The relative amount of *N*-oxide present in the sample was calculated as the measured concentration of the PA *N*-oxide divided by the sum of the concentration of PA *N*-oxide and the corresponding tertiary PA.

The same statistical test was used as for the chemical reduction experiment described above, to analyze if PA-structural group (senecionine, jacobine and erucifoline), reducing agent concentration (0, 0.01, 0.03, 0.1, 0.3, 1 M) and incubation time (1, 4, 24 h) did have a significant influence on the relative concentration of PA *N*-oxides formed by *N*-oxidation of the added tertiary PAs.

2.5. PA *N*-oxide reduction and PA *N*-oxidation in the presence of plant material

Three PA *N*-oxides (senecionine *N*-oxide, jacobine *N*-oxide and erucifoline *N*-oxide) and three tertiary PAs (senecionine, jacobine and erucifoline) were added separately to dry plant material of five different flowering *Asteraceae* species collected in the field, *S. gigantea*, *E. cannabinum*, *S. sylvaticus, J. erucifolia* (syn. *Senecio erucifolius*) and *J. vulgaris* (erucifoline chemotype). *S. gigantea* contains no PAs, *E. cannabinum* contains the lycopsamine type of PAs, *S. sylvaticus* contains the triangularine type of PAs, and *J. erucifolia* contains the senecionine type of PAs. Dried, ground plant material of the shoot (approximately 100 mg) was wetted with 500 µl distilled water containing senecionine *N*-oxide, jacobine *N*-oxide and erucifoline *N*-oxide (10 µg of each PA) in 3-fold and incubated for 1 h. As a control, from each plant species one sample was wetted with water containing no PA *N*-oxides. After adding 10 ml 2% formic acid and heliotrine as internal standard (1 µg/ml), the plant extract was shaken for 30 min. After centrifugation, the residual plant material was removed by filtering the extraction solution through a 0.2 µm nylon membrane. An aliquot of 25 µl filtered solution was diluted with 975 µl water and injected in the LC-MS/MS system.

2.6. Species description

J. vulgaris (syn. *Senecio jacobaea*) is a suitable system to study PAs. This species is native in Europe and West Asia but invasive in North America, Australia and New Zealand. In previous studies, up to 30 different PAs were detected in *J. vulgaris* (Witte et al, 1992; Macel et al, 2004; Kowalchuk et al, 2006; Joosten et al, 2009). Based on their structural features, major PAs in *J. vulgaris* can be divided into 3 structural groups: senecionine-like, comprising senecionine, integerrimine, retrorsine and (acetyl) seneciphylline; jacobine-like, comprising jacobine, jacoline, jaconine jacozine, and dehydrojaconine; erucifoline-like, comprising erucifoline and acetylerucifoline (Table 2).

Based on the PA composition, 4 chemotypes of *J. vulgaris* were distinguished: Senecionine-chemotype, largely lacking jacobine- and erucifoline-like PAs; Erucifoline-chemotype, lacking jacobine-like PAs; Jacobine-chemotype, containing high levels of jacobine-like PAs; mixed chemotype, containing both jacobine- and erucifoline-like PAs in similar amounts (Witte et al, 1992; Macel et al, 2004).

J. aquatica is a close relative but not a sister species to *J. vulgaris* (Pelser et al, 2003). These two species naturally hybridize in some areas and the hybrids can backcross into the parental populations (Kirk et al, 2004, 2005)

2.7. Effect of freeze-drying on the tertiary PA content

Freeze-drying is a general used method to dry plant material before analyzing PAs in plant material. In this way enzymatic activity can be prevented or at least strongly reduced. We tested if the freeze-drying can lead to spontaneous reduction of PAs. To compare freeze-dried material to the original plant condition we extracted PAs from fresh plant material as control treatment. Liquid nitrogen was used to ground fresh plant material under deep frozen conditions.

2.7.1. Plant material

One genotype of *J. vulgaris* originating from a population near Wageningen was used to study if reduction can take place during freeze-drying. The plants were propagated by tissue culture. In total eight clones per treatment (PA extraction of fresh material versus PA extraction of freeze-dried material) were used. The plants were potted in 1.3 l pots filled with potting soil (Slingerland Potgrond, Zoeterwoude, The Netherlands). The plants were kept in a climate room for 6 weeks (humidity 70%, light 16 h at 20 °C, dark 8 h at 20 °C) and randomly distributed every 8-10 days.

2.7.2. PA extraction from fresh and freeze-dried material

The shoot of each plant was cut lateral in two pieces with scissors so each part had an equal number of leaves of similar size, one shoot part for the control treatment and the other part for the freeze-drying treatment. The control part of the shoot was weighted, immediately ground under liquid N_2 and in frozen condition mixed in 20 ml of 2% formic acid containing 0.2 µg/ml heliotrine as internal standard. From this point on the standard PA extraction for LC-MS/MS was performed as described above. After weighting, the other half of the shoot was immediately stored at -20 °C before being freeze-dried. After freeze-drying, the standard PA extraction for LC-MS/MS was performed.

2.7.3. Data analysis

The 9 major PAs and their corresponding *N*-oxides were included in the analysis. We excluded the minor PAs which had a concentration close to detection limit and for which the ratios were not reliable. The relative concentration of tertiary amine (TA%) were calculated as: TA% = the tertiary amine concentration/(tertiary amine concentration + the corresponding *N*-oxide concentration) × 100. To calculate the percentage of *N*-oxides in fresh material transformed to tertiary amines during freeze-drying, the following formula was used to calculate the relative reduction amount of the *N*-oxides: (tertiary amine concentration in freeze-dried material–tertiary amine concentration in fresh material)/*N*-oxide concentration in fresh material. The difference of total PA, individual PAs and relative concentration of tertiary amines between the two methods were evaluated by paired *t*-test, with the absolute concentration of total PA, individual PA and TA% as the dependent variable, respectively. To test whether different individual PAs had a different amount of reduction from *N*-oxides to tertiary amines, a one-way ANOVA was performed with the relative reduction amount as variable and individual PA as group factor. All tests were conducted with SPSS 17.0 for Windows.

2.8 PA analysis for *J. vulgaris*

2.8.1. Plant material and PA analysis

Five different genotypes of *J. vulgaris* were used representing two chemotypes: three Jacobine-chemotypes and two Erucifoline-chemotypes. Two Jacobine-chemotypes originated from two different populations in Meijendel near The Hague and the third originated from a population near Wageningen. The two Erucifoline-chemotypes originated from a Dutch population near Vilt (Limburg) and a German population near Kassel. The five different genotypes were propagated by tissue culture. In total eight clones per genotype were used. The plants were potted in 1.3 l pots filled with calcareous sandy soil collected from Meijendel, a coastal dune area North of The Hague. The plants were kept in a climate room for 5 weeks (humidity 70%, light 16 h at 20 °C, dark 8 h at 20 °C) and randomly distributed every 8-10 days.

After 5 weeks the plants were harvested in order to determine the PA concentration and composition. The plants were cut with scissors just above the root crown and roots and shoots were immediately stored at -20 °C for 4 days before being freeze-dried for 1 week under vacuum with a collector temperature of -55 °C (Labconco Free Zone® 12 l Freeze Dry System). PAs were extracted by formic acid, as described above. An aliquot of 25 µl filtered solution was diluted with 975 µl water and injected in the LC-MS/MS system.

2.8.2. Data analysis

A two-way ANOVA was used to analyze if chemotype and plant part (root and shoot) have a significant influence on the TA%. The ANOVA was performed by GLM (General Linear Model) univariate analyses procedure with TA% as the dependent variable, chemotype and plant part as fixed factors. The tests were conducted with SPSS 17.0 for Windows.

2.9. Relative concentration of tertiary amine analysis for *Jacobaea* hybrids

2.9.1. Plant material and PA analysis

F_2 hybrids of two different species were used in this study; *J. vulgaris* subs. *dunensis* and *J. aquatica* subs. *aquatica*. Seeds were collected for *J. vulgaris* at Meijendel, a coastal dune area north of The Hague (The Netherlands) and for *J. aquatica*, a coastal dune area at Zwanenwater Reserve (The Netherlands). Crossings were performed by rubbing flower heads together. This cross resulted in numerous seeds which were germinated. Both species are self incompatible and all F_1 and F_2 seeds are true crosses confirmed by molecular analysis (unpublished data). Two F_1 individuals with rayed flowers were chosen and crossed reciprocally with each other resulting in offspring. The two parental, two F_1 and >100 F_2 individuals were maintained in tissue culture.

The plants used in this study were cloned from the tissue culture material. Beside the two parental genotypes (*J. vulgaris* and *J. aquatica*) and two different F_1 hybrids, 102 different F_2 hybrid genotypes were used. On average 6 cloned replicates per F_2 genotype and 12 cloned replicates per parental and F_1 genotype were grown. In total, 609 plants were used in this study, among which 562 were F_2 individuals.

The plants were potted in 1.3 l pots filled with 95% sandy soil, collected from Meijendel, 5% potting soil (Slingerland Potgrond, Zoeterwoude, The Netherlands) and 1.5 g/l Osmocote (Scotts®, Geldermalsen, The Netherlands, N:P:K = 15:9:11). The plants were randomly distributed and kept in a climate room for 6 weeks (humidity 70%, light 16 h at 20 °C, dark 8 h at 20 °C). After 6 weeks the plants were harvested and prepared for LC-MS/MS analysis as described above.

2.9.2 Data analysis

Of the 37 detected PAs, 9 were otonecine structural group PAs for which no corresponding *N*-oxide exists and 6 were absent or close to the detection limit in some samples. The remaining 22 PAs were used to calculate the relative concentration of tertiary amine as TA% = the tertiary amine concentration/(tertiary amine concentration + the corresponding *N*-oxide concentration) × 100.

The genotype effect on TA% was statistically analyzed by a Kruskal-Wallis test with the TA% as the independent variable and genotype (including parental, F_1 and F_2, 106 genotypes in total) as the grouping variable. Spearman correlation matrix between the 11 kinds of TA% was calculated based on the mean TA% per genotype in root and shoot. *P*-values of the correlations were adjusted by Holm's method (Holm, 1979). To determine if different type of plant material (root/shoot) had a different degree of the correlation between TA%, a paired *t*-test was done with the correlation values as the independent variable.

Depending on the PA-structural group the specific PAs belong to, the correlations were divided into 6 categories: Category 1, correlation between the PAs of the senecionine-like PAs; Category 2, correlation between the PAs of the jacobine-like PAs; Category 3, correlation between the PAs of

the erucifoline-like PAs; Category 4, correlation between the PAs of the senecionine- and jacobine-like PAs; Category 5, correlation between the PAs of the senecionine- and the erucifoline-like PAs; Category 6, correlation between the PAs of the jacobine- and the erucifoline-like PAs. Differences between the correlation values belonging to the different categories were analyzed with a one-way ANOVA with the correlation values as the independent variable and correlation category (Category 1-6) as fixed factor. All tests were conducted with SPSS 17.0 for Windows, except for the correlation matrix and adjustment by Holm's method, which was conducted with R 2.10.0 for Windows.

3. Results

3.1. Chemical reduction of PA *N*-oxides

The chemical reduction of the three PA *N*-oxides, senecionine *N*-oxide, jacobine *N*-oxide and erucifoline *N*-oxide, with sodium metabisulfite into their tertiary amines showed a significant difference ($F_{2, 87}$ = 10.8, $P < 0.001$) in rate of reduction at any concentration of sodium metabisulfite added. Averaged over all incubation times (1, 4, 24 h) and reducing agent concentrations (0.01, 0.03, 0.1, 0.3, 1 mM) 42.2% (SE ± 0.63) of the jacobine *N*-oxide was reduced while 45.8% (SE ± 0.63) for both senecionine *N*-oxide and erucifoline *N*-oxide (Fig.S1). However, the difference is not significant due to the analytical error, which is estimated at 10%.

Exposure of the three PA *N*-oxides to 1 M cysteine produced no measurable amount of tertiary amines after 24 h under acidic conditions (2% formic acid). However, under neutral conditions (water) with 1 M cysteine a very slow reduction occurred: after 24 h the production of senecionine, jacobine and erucifoline was respectively 1.9%, 4.2% and 2.7% (data not shown). The amounts of tertiary amines formed were too low to draw definitive conclusions about a difference in reactivity of the PA *N*-oxides towards cysteine and other potential sulfur-containing plant components. It should be pointed out that under the extraction conditions used in this study, the PA *N*-oxides displayed no measurable reactivity whatsoever towards cysteine. Interestingly, we found that 1 M cysteine catalyzed the isomerisation of senecionine *N*-oxide into integerrimine *N*-oxide notably under acidic conditions. After 24 h approximately 30% of senecionine *N*-oxide has isomerised to integerrimine *N*-oxide, under neutral condition this was only 14%. In the absence of cysteine the isomerisation in formic acid was less than 1% after 24 h.

3.2. Chemical *N*-oxidation of tertiary PAs

For the chemical oxidation under acidic conditions of the three macrocyclic tertiary PAs, senecionine, jacobine and erucifoline, with hydrogen peroxide (HOOH) into their *N*-oxides, relatively high concentrations of peroxide were required to induce oxidation at a measurable rate. Oxidation with HOOH proceeded much faster under neutral conditions (data not shown). Averaged over all incubation times (1, 4, 24 h) and oxidation agent concentrations (0.01, 0.03, 0.1, 0.3, 1 mM), 2.8% (SE ± 0.14) of the jacobine was oxidized while 1.0 (SE ± 0.14) and 1.1% (SE ± 0.14) for senecionine and erucifoline, respectively. The chemical oxidation of senecionine and erucifoline takes place with approximately the same rate, but that the oxidation of jacobine proceeded significantly faster ($F_{2, 105}$ = 48.6, $P < 0.001$). The difference in rate was irrespective to the HOOH concentration. After 24 h with 1 M peroxide approximately 22.2% (SE ± 1.5) of jacobine had been converted to its *N*-oxide, while for senecionine the conversion was only 6.4% (SE ± 1.5) and for erucifoline 7.5% (SE ± 1.5) (Fig.S2).

3.3. Extraction of tertiary PAs and PA *N*-oxides in the presence of dried plant material of five different Asteraceae species

The three PA *N*-oxides, senecionine *N*-oxide, jacobine *N*-oxide and erucifoline *N*-oxide, in presence of dry plant material of 5 flowering *Asteraceae* species showed no measurable induced formation of tertiary amine PAs by naturally reducing agents if present (data not shown). All PA *N*-oxides added were recovered with LC-MS/MS after extraction. Only a very small amount (2%) of the added senecionine *N*-oxide was reduced in the presence of *Solidago gigantea* and *Eupatorium cannabium* plant material, but the concentrations measured were close to the detection limit. In the presence of *Senecio sylvaticus* no reduction was observed for all three PA *N*-oxides. In the control samples (no PA *N*-oxides added) of *Jacobaea erucifolia* and *J. vulgaris* senecionine *N*-oxide, erucifoline *N*-oxide and its tertiary PAs were already present in the plant material but jacobine or jacobine *N*-oxide were not present in detectable amounts. Since senecionine *N*-oxide and erucifoline *N*-oxide were naturally present in the plant, we could not draw any conclusions on the reduction of these PAs, as the added *N*-oxide volumes were negligible. For the jacobine *N*-oxide added it could be shown that there was no reduction by naturally occurring reducing agents present in *J. erucifolia* and *J. vulgaris*.

The three tertiary PAs, senecionine, jacobine and erucifoline, in presence of dry plant material of several flowering *Asteraceae* species showed no detectable induced oxidation of PAs by naturally occurring oxidation agents (data not shown). All PAs added were recovered after extraction.

3.4. Effect of freeze-drying on the tertiary PA content

The total PA concentration and the concentration of the individual PAs was not significantly different comparing the freeze-dried with fresh plant material (Table 1). The freeze-dried (lyophilized) materials had a higher TA% for all individual PAs compared to the corresponding fresh materials, which illustrates that the freeze-drying process caused some reduction from *N*-oxide to tertiary amine. The reduction is not PA specific, because the relative reduction amount was not significantly different between the PAs (Table 1, ANOVA, $F_{8, 63}$ = 0.69, P = 0.70).

Table 1 Effect of sample treatment on the observed concentration of total PA, individual PA, relative concentration of tertiary amines (TA%), and relative reduction amount.

PA[a]	Concentration[b] (mg/g dry wt)			TA%[c]			Relative reduction amount[d] (%)
	Freeze-dried	Fresh	Paired t- test	Freeze-dried	Fresh	Paired t- test	
total PA	0.654	0.794	ns	22	13	*	4
sn	0.042	0.047	ns	6	1	*	3
ir	0.014	0.017	ns	5	1	*	2
sp	0.095	0.108	ns	7	2	*	3
acsp	0.012	0.008	ns	4	2	*	5
jb	0.435	0.560	ns	28	17	**	4
jl	0.007	0.008	ns	43	29	*	12
jz	0.011	0.011	ns	20	11	ns	6
er	0.020	0.020	ns	9	3	ns	6
acer	0.014	0.014	ns	5	1	*	3

[a] Abbreviations: sn = senecionine; ir = integerrimine; sp = seneciphylline; acsp = acetylseneciphylline; jb = jacobine; jl = jacoline; jz = jacozine; er = erucifoline; acer = acetylerucifoline
[b] Concentration was the absolute concentration of PAs as tertiary amines and *N*-oxides
[c] TA% = the tertiary amine concentration/(tertiary amine concentration + the corresponding *N*-oxide concentration) × 100.
[d] Relative reduction amount = (concentration of tertiary amines in freeze-dried material – concentration of tertiary amines in fresh material)/ concentration of the corresponding *N*-oxides in fresh material.
ns: not significant, * P < 0.05, ** P < 0.01, *** P < 0.001)

3.5. PA distribution in *Jacobaea vulgaris*

A total of 27 different PAs (*N*-oxides + tertiary amines) were found in roots and shoots of the five genotypes. Dehydrojaconine, spartioidine and senecivernine were found in trace amounts and did only occur in detectable amounts as tertiary PA, while all other individual PAs were found in both forms.

The mean TA% in the roots of Jacobine-chemotypes and both plant parts of Erucifoline-chemotypes were all below 6.2%, while the TA% in the shoots of Jacobine-chemotypes was approx. 6 times higher, resulting in a significant chemotype $_{x}$ plant part interaction (ANOVA, $F_{1, 78} = 53.07$, $P < 0.001$). In the roots no significant difference between the chemotypes (Mean TA% roots Jacobine and Erucifoline-chemotype = 5.3% and 5.7%, respectively) was found while in the shoots the difference was highly significant (Mean TA% shoots Jacobine and Erucifoline-chemotype = 37.0% and 6.1%, respectively).

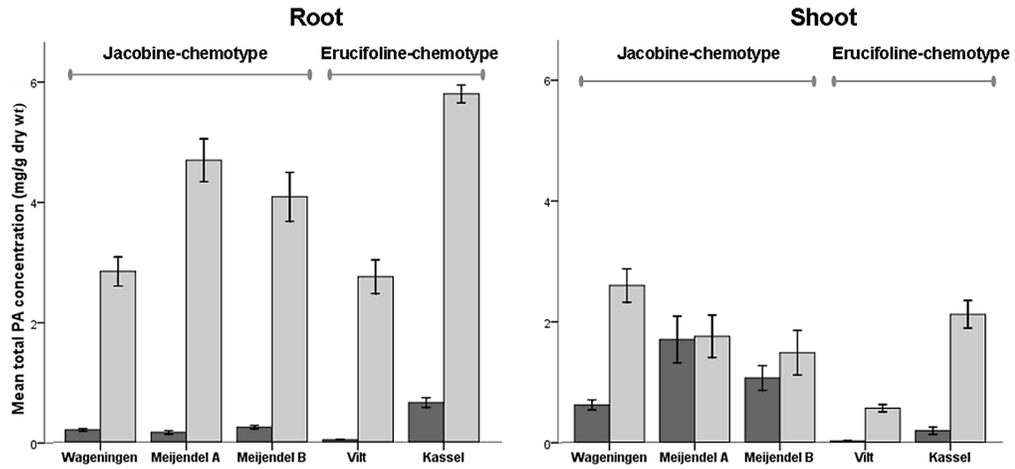

Fig.1 The absolute mean total PA concentration in dry root and shoot material per genotype (n = 8). Light bar = PA *N*-oxides and dark bar = tertiary PAs. Error bars: ±1SE. Above the bars, the genotype the chemotype is indicated.

In the roots of all genotypes on average 94.7% of all PAs were in *N*-oxide form (Fig.1). Senecionine *N*-oxide, seneciphylline *N*-oxide and acetylseneciphylline *N*-oxide were the most abundant PAs in the roots with on average 71.0% of the total PA root concentration (Fig.4). The Jacobine-chemotypes from Meijendel (Meijendel A and B) contained jacobine *N*-oxide as one of the dominant root PAs, while the Erucifoline-chemotypes (Vilt and Kassel) contained erucifoline *N*-oxide as a dominant PA (Fig.2), with respectively 14.3% (for jacobine) and 14.9% (for erucifoline) of the total PA root concentration.

The four most dominant PAs in the shoots of the Erucifoline-chemotypes were senecionine, seneciphylline, erucifoline and acetylerucifoline. In the shoots of this chemotype, a lower concentration of PAs were in the tertiary PA form as compared to the Jacobine-chemotypes with only 3.6% and 8.2% of the total shoot PA concentration for Vilt and Kassel, respectively (Fig.1).

The TA% in the shoots was higher in the Jacobine-chemotypes. In particular, the chemotypes from Meijendel contained a high percentage of tertiary PAs (Fig.1). In the shoots of this chemotype, on average 45.5% of the total shoot PA concentration occurred as tertiary PA. In the Jacobine-chemotype from Wageningen, tertiary forms comprised nearly 20% of the total shoot PA concentration (Fig.1).

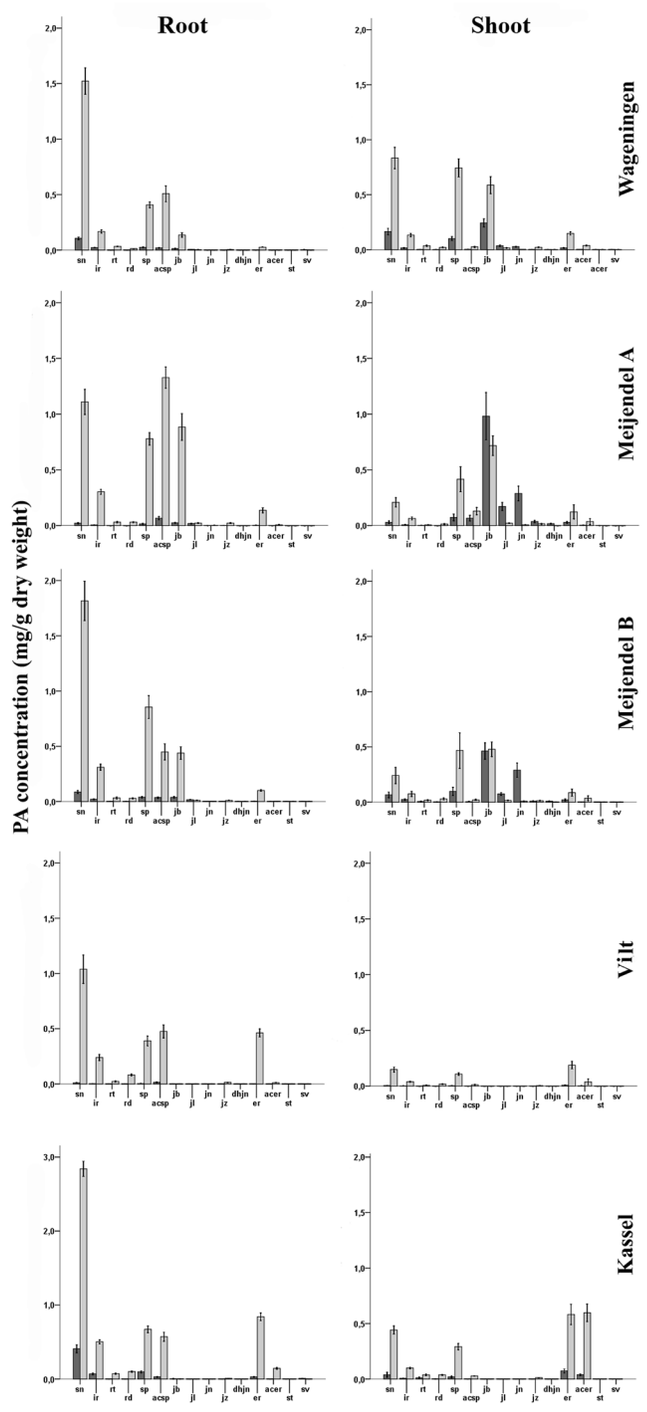

Fig.2 The PA composition of *J. vulgaris* in absolute concentration per individual PA of dry root and shoot material (n = 8) Light bar = PA *N*-oxides and dark bar = tertiary PAs. For abbreviations see legend Table 2. Error bars: ±1SE.

The TA% is in fact only determined by the presence of the jacobine-like PAs. Jacobine and its derivatives jaconine, jacoline, jacozine and dehydrojaconine showed the highest percentage in reduced form (Fig.2). In the two Jacobine-chemotypes from Meijendel on average only 17.0% of the total senecionine and seneciphylline concentration was present as tertiary PAs while for jacobine this was 54.1%.

3.6. Relative tertiary amine concentration in *Jacobaea* hybrids

Of the 37 detected PAs in the *Jacobaea* hybrids, 9 were otonecine-group PAs with no corresponding *N*-oxides and 6 were absent or close to the detection limit in some samples. The remaining 22 PAs were used to calculate the relative concentration of tertiary amine as TA%.

The TA% of the senecionine-like and erucifoline-like PAs in the roots were lower than 10%, which demonstrates that more than 90% of these PAs were present in *N*-oxide in the roots. But the jacobine-like PAs had TA% ranging from 10% till 56%. Except for senecionine, integerrimine and acetylerucifoline, the TA% of all the other PAs was genotype dependent in the roots. In the shoots, the TA% were higher than those in the roots (for all 11 PAs, paired *t*-test, $df = 608$, $P < 0.001$). Particularly for jaconine, the TA% was up to 80% in the shoots. The TA% of all the individual PAs were genotype dependent in the shoots (Table 2).

Generally there was a significant positive correlation between the TA% both in the roots and in the shoots. The correlation coefficients were not significantly different between the shoots and roots (paired *t*-test, $df = 54$, $t = -0.393$, $P = 0.696$), but correlation coefficients differed between structural groups (ANOVA, $F_{5, 104} = 10.69$, $P < 0.001$). Correlation coefficients of TA% within structural groups are always higher than TA% correlation between different structural groups (Fig.S3).

Table 2 The concentration of tertiary and *N*-oxide PA, TA% and the genotype effect on the TA% in two parental, two F1 and 102 F2 genotypes from a cross between *J. vulgaris* and *J. aquatica*.

Plant Part	Structural group	Pyrrolizidine alkaloid	Code	Concentration (mg/g dry wt) Tertiary amine	-oxide	TA%[a]	x^{2} [b]	P[c]
Roots	Senecionine-like	senecionine	sn	0.053	1.435	4	111.7	ns
		intergerrimine	ir	0.007	0.232	3	97.9	ns
		retrorsine	rt	0.002	0.037	5	131.8	*
		seneciphylline	sp	0.025	0.601	4	144.6	**
		acetylseneciphylline	acsp	0.047	0.996	5	133.9	*
	Jacobine-like	jacobine	jb	0.029	0.250	13	245.2	***
		jacoline	jl	0.009	0.013	45	252.1	***
		jaconine	jn	0.033	0.025	56	166.7	***
		jacozine	jz	0.001	0.009	10	268.2	***
	Erucifoline-like	erucifoline	er	0.003	0.039	9	144.5	**
		acetylerucifoline	acer	0.000	0.009	6	98.4	ns
Shoots	Senecionine-like	senecionine	sn	0.011	0.177	8	144.2	**
		integerrimine	ir	0.003	0.063	7	132.6	**
		retrorsine	rt	0.001	0.009	17	163.6	***
		seneciphylline	sp	0.038	0.513	9	134.2	**
		acetylseneciphylline	acsp	0.009	0.148	10	147.5	**
	Jacobine-like	jacobine	jb	0.077	0.234	24	311.9	***
		jacoline	jl	0.023	0.016	55	354.8	**
		jaconine	jn	0.252	0.047	80	376.4	***
		jacozine	jz	0.003	0.012	31	343.3	***
	Erucifoline-like	erucifoline	er	0.015	0.138	15	203.5	***
		acetylerucifoline	acer	0.004	0.052	11	134.9	*

[a] TA% = the tertiary amine concentration/(tertiary amine concentration + the corresponding *N*-oxide concentration) × 100.
[b] Kruskal-Wallis test with the concentration data from two parental, two F1 and 102 F2 genotypes. Ca. 12 replicates per parental and F1 and ca. 6 replicates per F2 hybrid, in total n = 609 plants.
[c] ns: not significant, * P < 0.05, ** P < 0.01, *** P < 0.001

4. Discussion

We observed that the tertiary amine proportion was different among PAs and genotypes. Two possible and nonexclusive hypotheses may explain this pattern. Firstly, the chemical transformation and perhaps allocation of PA *N*-oxides, is accompanied by a continuous slow reduction of the original *N*-oxides. Thus, the most peripheral "on a time scale oldest" PAs like jacoline and jaconine, which are far down the pathway (Fig.S4), show the highest TA% and the "youngest" PAs, i.e. senecionine or intergerrimine, have the lowest TA%. The observation that the TA% values in shoots are always higher than the values for the respective PAs in roots goes in the same direction (Hartmann, 2010, personal communication). Secondly, specific (re-)oxidation of the tertiary PAs might explain the pattern. The reduction of PA *N*-oxides in the plant is an unspecific, chemical process induced by the presence of endogenous reducing compounds and (traces of) transition metal salts. Meanwhile, there is a, biochemically based, process operating to re-oxidize the reduced tertiary amines for PA transport. Enzyme(s) that may be involved seem to work well for senecionine-like and erucifoline-like PAs but work less well for jacobine-like PAs. Possibly, the substrate specific enzyme is affected when alterations at positions 15 and 20 (addition of O, H_2O, HCl, Fig.S5) are made. This perhaps makes the epoxidized PAs less accessible for the enzyme, which results in a lower conversion rate. So, the second hypothesis could explain the TA% difference among the PAs and the genotypes. Furthermore, it may get more support from a biochemical point of view, since the plant has to use an enzyme to produce the backbone senecionine *N*-oxide at the beginning of the PA pathway. Senecionine *N*-oxygenase (SNO) was isolated (from the larvae of specialist insect *Tyria jacobaeae*, less relevant for plants) and *Crotalaria scassellatii* seedlings. The enzymes were tested with different PAs as substrates and showed that they specifically oxidized tertiary PAs (Lindigkeit et al, 1997; Chang and Hartmann, 1998). These enzymes might be highly preserved and similar in the various PA containing plants. A very interesting follow-up of this study could be the identification, isolation and characterization of this putative *N*-oxidation enzyme(s) and exploration of genetic variation concerning these enzymes. It would also be interesting to see if the TA% can be influenced by external factors, like a high metal content in the soil, or by application of reducing compounds to the leaves.

Our results showed that jacobine-like PAs had a higher TA% than the other PAs. This coincides with the role of jacobine-like PAs as important defense compounds. Several studies showed that jacobine and jaconine were especially feeding deterrent for generalist insect herbivores (Macel et al, 2005; Leiss et al, 2009), while some specialists, preferred plants containing high concentrations of jacobine (Macel and Klinkhamer, 2010). From an evolutionary and ecological point view, it represents a next step in the arm-race between plants and herbivores as a number of studies show that tertiary amines are more toxic than their respective *N*-oxides (Dreyer et al, 1985; van Dam et al, 1995; Macel et al, 2005). Further research on the chemistry and biology of PA *N*-oxides and tertiary PAs and their influence on generalist and specialist insects are required to better understand the ecological significance of these highly interesting compounds.

We showed that the occurrence of tertiary PAs is not an artifact of the freeze drying, extraction or detection method. The three main PA *N*-oxides of *J. vulgaris* showed no significant differences during the reduction experiments. Jacobine was significantly more reactive compared to senecionine and erucifoline towards chemical *N*-oxidation with oxidation agent HOOH.

These results strongly indicate that the high levels of free bases found for jacobine and other

jacobine-like PAs are not caused by an intrinsic structural instability of the PA molecule or by chemical attack. Also it was not observed that naturally occurring agents in plant material caused reduction or oxidation of the added PAs during our extraction method. Plant material of different species did not induce any transformation of PAs from one form into the other. From our results we can conclude that the high percentages in tertiary form for jacobine-like PAs are not due to instability or higher sensitivity for reducing agents in the extraction and analytical process, but likely are the result of a change induced by (bio) chemical processes in the plant itself. We cannot exclude that a minor amount of reduction occurs during harvesting and the freeze-drying, but it seemed to affect all PA *N*-oxides to the same extent. We did find that in the Jacobine-chemotype plants a much higher level of tertiary PA present compared to the Erucifoline-chemotypes. By crossing *J. vulgaris* Jacobine-chemotype with the closely related *J. aquatica*, which lacks jacobine, and measuring PA *N*-oxide and tertiary amine concentrations, we showed that the TA% was genotype-dependent. This means that the variation found for relative tertiary amine content has a genetic base, since the environmental conditions of the plants during growth and analysis were kept equal for all plants.

Acknowledgements
We are grateful to Kelly Stolk, Anniek de Vreede, Cilke Hermans, Karin van der Veen-van Wijk, Ciska Raaijmakers and Henk Nell for their technical assistance. We thank Prof. Dr. Thomas Hartmann for his helpful comments on the manuscript and the hypothesis on slow unspecific reduction of PA *N*-oxides. We thank the China Scholarship Council (CSC) of the Ministry of Education for financial support.

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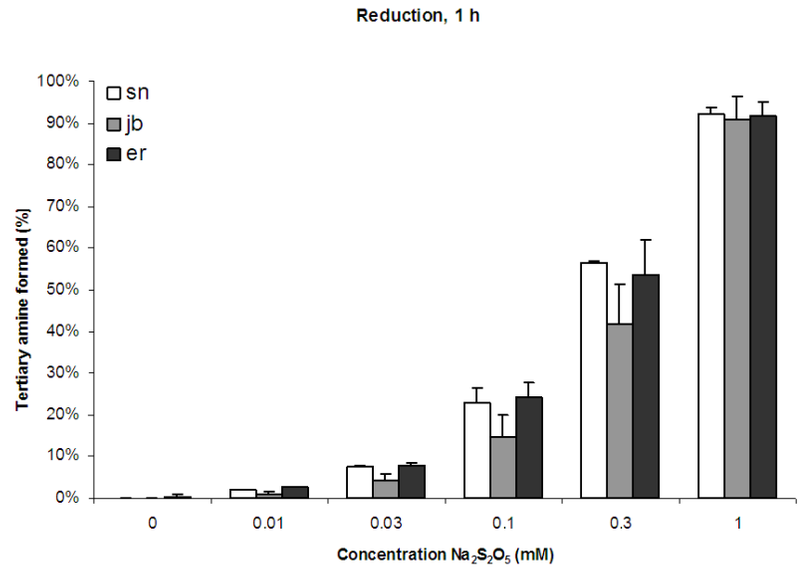
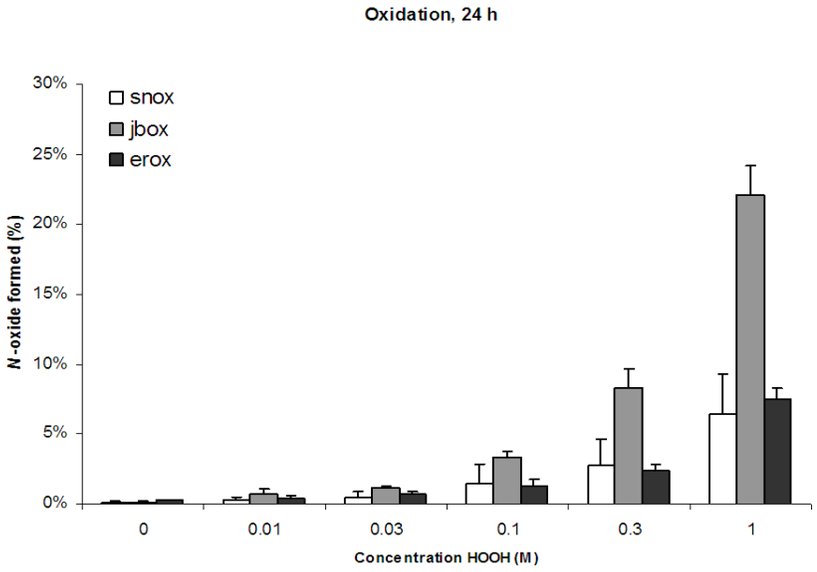
Supplementary Material



- **Fig.S1** Reduction of PA *N*-oxides after incubation (1h, n = 2) in 2% formic acid solution with sodium metabisulfite ($Na_2S_2O_5$) in five different concentrations (0.01, 0.03, 0.1, 0.3, 1 mM). sn = senecionine; jb = jacobine; er = erucifoline. Error bars: ±1SD. The tertiary amine formed is the relative amount of tertiary PA present in a sample, which was calculated as the measured concentration of tertiary PA divided by the sum of the concentration of tertiary PA and corresponding PA *N*-oxide.



- **Fig.S2** Oxidation of PA tertiary amines after incubation (24h, n = 2) in 2% formic acid solution with hydrogen peroxide (HOOH) in 5 different concentrations (0.01, 0.03, 0.1, 0.3, 1 M). snox = senecionine *N*-oxide, jbox = jacobine *N*-oxide, erox = erucifoline *N*-oxide. Error bars: ±1SD. The *N*-oxide formed is the relative amount of *N*-oxide present in the sample which was calculated as the measured concentration of the PA *N*-oxide divided by the sum of the concentration of PA *N*-oxide and the corresponding tertiary PA.

sn	0.85***	0.38**	0.79***	0.68***	0.25ns	0.17 ns	0.17 ns	0.15 ns	0.45***	0.46***
0.89***	ir	0.35**	0.77***	0.60***	0.32*	0.21 ns	0.21 ns	0.06 ns	0.46***	0.44***
0.55***	0.55***	rt	0.42***	0.38***	0.37**	0.35**	0.40***	0.48***	0.55***	0.49***
0.83***	0.80***	0.58***	sp	0.66***	0.35**	0.23 ns	0.24 ns	0.29*	0.68***	0.63***
0.81***	0.84***	0.56***	0.80***	acsp	0.27*	0.28*	0.29*	0.34**	0.5***	0.59***
0.43***	0.38**	0.32*	0.51***	0.50***	jb	0.82***	0.86***	0.46***	0.6***	0.44***
0.43***	0.37**	0.26 ns	0.52***	0.49***	0.80***	jl	0.87***	0.49***	0.56***	0.48***
0.51***	0.43***	0.34**	0.54***	0.55***	0.79***	0.82***	jn	0.53***	0.59***	0.49***
0.15 ns	0.10 ns	0.31*	0.20 ns	0.18 ns	0.19 ns	0.07 ns	0.17 ns	jz	0.58***	0.43***
0.50***	0.41***	0.51***	0.52***	0.44***	0.43***	0.45***	0.52***	0.31*	er	0.75***
0.21 ns	0.18 ns	0.14 ns	0.15 ns	0.15 ns	0.02 ns	0.03 ns	0.17 ns	0.28 ns	0.21 ns	acer

- **Fig.S3** The correlations between genotype mean TA% of the PAs. Two parental, two F1 and 102 F2 genotypes were used. Numbers in the cells are the correlation coefficients. The background color of the cells is related to the number: black (>0.75); dark grey (0.50~0.75); light grey (0.25 ~ 0.50); white (< 0.20 and (or) p-value is not significant). ns: not significant, * p<0.05,**p<0.01, ***p<0.001. In the cells along the diagonal line are the codes for PAs. Sn = senecionine; ir = integerrimine; rt = retrorsine; sp = seneciphylline; acsp = acetylseneciphylline; jb = jacobine; jl = jacoline; jz = jacozine; er = erucifoline; acer = acetylerucifoline. Correlation coefficients above the diagonal line are for shoots, below the diagonal for roots.

- **Fig.S4,5** are Appendix 1-2 at the end of this thesis

Chapter 4

The influence of pyrrolizidine alkaloid variation on cinnabar moth oviposition preference in *Jacobaea* hybrids

Dandan Cheng, Eddy van der Meijden, Klaas Vrieling, Patrick P.J. Mulder, Peter G.L. Klinkhamer

Specialist herbivores may use the secondary metabolites produced by their host plants for host recognition, oviposition and feeding stimulation or to their own defense against parasites and predators. Still an open question is whether specialist herbivores are a selective force in the evolution of the great diversity of plant secondary metabolites. A prerequisite for such a selective force would be that the preference and (or) performance of specialist herbivores is influenced by plant secondary metabolites.

The cinnabar moth (*Tyria jacobaeae*) is one of the main specialist herbivores of *Jacobaea vulgaris* and is adapted to pyrrolizidine alkaloids (PAs), the defense secondary metabolites in its host plants. To investigate whether oviposition preference of cinnabar moths is affected by PAs, we conducted an oviposition experiment with cinnabar moths using 40 tissue culture cloned F_2 genotypes of an artificial hybrid family of *Jacobaea vulgaris* and *Jacobaea aquatica*.

We found that the number of eggs and the number of egg batches oviposited by the cinnabar moths were dependent on plant genotypes and cinnabar moth oviposition preference was positively correlated to the concentration of tertiary amines of jacobine-like PAs and some otosenine-like PAs. Synergy was found between the effects of jacobine-like and otosenine-like PAs on oviposition preference. The PAs from the other two PA groups (senecionine- and erucifoline-like PAs) did not relate to oviposition preference. Our results suggest PAs in host plant influence the cinnabar moth oviposition preference and this insect is a potential selective agent on the concentration of some individual PAs.

Key Words: Secondary metabolites, diversity, host plant choice, specialist herbivores, chemical defense

1. Introduction

Plants produce a vast variety of structurally different secondary metabolites (De Luca and St Pierre, 2000). Secondary metabolites (SMs) mainly function as defense against antagonistic organisms and/or as signal chemicals for communication with potentially beneficial organisms. In addition they often play a role in protection against abiotic stresses (see reviews by Wink, 2003; Hartmann, 2007). Within a particular species, or an individual plant, a few major compounds are usually accompanied by several derivatives as minor components (Wink, 2003), Beside the structural diversity, SMs often show great variation in concentration. It has been demonstrated that the SM variation in regard to composition and concentration is under genetic control (Vrieling et al, 1993; van Dam and Vrieling, 1994; Kliebenstein et al, 2001, Lankau, 2007).

Herbivores are thought to play an important role in the evolution of the SM diversity in plants (Ehrlich and Raven, 1964; van der Meijden, 1996; Futuyma and Agrawal, 2009). Specialist herbivores usually adapt to a class of defense compounds in a host plant, use them as oviposition and feeding cues, and even utilize them for their own defense (Schoonhoven et al, 2005). Therefore, specialist herbivores have been regarded as being less affected by a given chemical defense than the generalist herbivores and are unlikely to be a selective force in the evolution of a group of structurally related SMs (Harvey et al, 2005; Macel et al, 2005; Arany et al, 2008), However, structurally related compounds can have different simulating effects on specialist herbivores (Macel and Vrieling, 2003) and the variation of defense chemicals in host plants may affect the specialist herbivores' preference (Nieminen et al, 2003;Leima et al, 2005). Moreover, specialist herbivores can exert selection on the concentration of defense chemicals. For instance, the field work manipulating specialist and generalist herbivores of *Brassica nigra* independently showed that specialist loads were positively correlated with increasing sinigrin concentrations in *B. nigra* and higher sinigrin concentration was favored when specialists were removed (Lankau, 2007).

Jacobaea species, formerly known as *Senecio* species, are a good model system to study the diversity of a single group of SMs in plants. These species contain a diverse but structurally related group of PAs that play a role in interactions between plants and their herbivores and pathogens (Hol and van Veen, 2002; Macel et al, 2005; Kowalchuk et al, 2006, Joosten et al, 2009). PAs can occur in plants in two forms: tertiary amine (free base) and *N*-oxide (Rizk, 1991; Wiedenfeld et al, 2008; Chapter 3). Twenty-six different PAs (as tertiary amines) have been reported from 24 *Jacobaea* species (Pelser et al, 2005) using gas chromatography (GC). However, recently more sensitive analytical methods such as liquid chromatography-tandem mass spectrometry (LC-MS/MS) detected 37 structural PA variants even within a single species (Chapter 2-3). The effects of single PAs on PA-unadapted generalist insect herbivores and nematodes are dependent on PA structure and concentration (van Dam et al, 1995; Macel et al, 2005; Dominguez et al, 2008; Thoden et al, 2009). The two forms of the same individual PA had different deterring effect on non-adapted generalist insect herbivores from the results of *in-vitro* bioassay with isolated PAs (Dreyer et al, 1985; van Dam et al, 1995; Macel et al, 2005). The simulative effects of PAs' on oviposition and feeding of specialist were confirmed by bioassays with isolated PAs but it is still largely unknown whether PAs in host plants affect preference of specialist insects (Macel, 2011). Adapted insects are capable of *N*-oxidation of tertiary amines formed in the gut and subsequently store these PA *N*-oxides in their body (Hartmann, 1999).

The cinnabar moth (*Tyria jacobaeae*) is a specialist arctiid moth that mainly feeds on *Jacobaea vulgaris* (syn. *Senecio jacobaea*) and a restricted number of other *Senecio/Jacobaea* species. *Tyria jacobaeae* sequesters and metabolizes PAs for its own defense (Rothschild et al, 1979; van Zoelen and van der Meijden, 1991; Lindigkeit et al, 1997; Naumann et al, 2002). Experiments with artificial leaves lined with PAs showed that PAs are oviposition stimulants for the cinnabar moth and that the stimulatory effects differ among the particular PAs (Macel and Vrieling, 2003). However, some studies showed that the adult oviposition preference and larval performance of the cinnabar moth was not related to the PAs in host plants (Vrieling and de Boer, 1999; Macel et al, 2002) and oviposition host plant choice among the plants of *J. vulgaris* were related to other factors such as sugar and nitrogen (van der Meijden et al, 1989). Macel and Klinkhamer (2010) found that the damage on *J. vulgaris* plants was mainly caused by specialist insect herbivores such as *T. jacobaeae*, *Longitarsus jacobaeae* and *Haplothrips senecionis*, and that herbivory was positively correlated to the concentration of total PAs and individual PAs (jacobine and jacobine *N*-oxide). *Jacobaea vulgaris* in invasive areas (where it is free of specialist insect herbivore attack) contained higher amounts of PAs compared to those in native areas. In addition, the *J. vulgaris* plants in the invasive areas contain jacobine as the major PA (Joshi and Vrieling, 2005).The previous studies mentioned above seemed to be contradictory and it is not clearly yet whether plant PA variation affect cinnabar moth oviposition preference. To answer this question, we designed a controlled oviposition bioassay with cinnabar moths on the plants of different F_2 hybrid genotypes from a cross between *J. vulgaris* and *Jacobaea aquatica*. Segregating hybrid plants demonstrated greater ecological and chemical variation compared to parental species (Fritz, 1999; Orians, 2000; Kirk et al, 2011) and the various traits are expected to be independent from one another except if they are linked. Therefore they are regarded as useful tools to study the relation between different traits in plants (Hochwender et al, 2000; Orians, 2000; Lexer et al, 2003; Orians et al, 2010). We found in a previous study that PA composition and concentration varied widely between the F_2 hybrids of *J. vulgaris* and *J. aquatica* (Chapter 2). In this study, we address the following questions: 1) Do the cinnabar moths have an oviposition preference for certain hybrid plant genotypes? 2) Is oviposition preference affected by the concentration of total PA and of individual PAs in host plants? 3) Is oviposition preference affected by the synergistic or antagonistic effects between PAs?

2. Methods and Material

2.1. Plants grown for the oviposition bioassay

The plants used in the oviposition bioassay were from a hybrid family stored in tissue culture. The hybrid family consists of two parental, two F_1 and 102 F_2 individuals, which were cloned in order to obtain replicate individuals of a genotype for the experiments described here. Such a set of cloned individuals are referred to as 'genotypes' hereafter. The parental genotypes are a jacobine-chemotype plant of *J. vulgaris* and a *J. aquatica* plant. The *J. vulgaris* genotype is from a seed collected at Meijendel Nature Reserve (52° 7′ 54″ N, 4° 19′ 46″ E, The Netherlands) and *J. aquatica* genotype is from a seed collected at the Zwanenwater Reserve (52° 48′ 38″ N, 4° 41′ 7″ E, The Netherlands) (see more details of this hybrid system in Chapter 2). Forty F_2 hybrids genotypes were selected from the hybrid system according to PA composition and concentration in their shoot (Chapter 2). We selected genotypes with a large range in concentration of total PA and major PAs such as senecionine, jacobine and erucifoline (for both the tertiary amine and the *N*-oxide form).

The 40 F_2 genotypes were propagated by tissue culture. Plants were potted in 1.3 liter pots (ca.9 cm diameter, 9 cm high) filled with a mixture of 95% sandy soil from Meijendel, 5% potting soil (Slingerland Potgrond company, Zoeterwoude, The Netherlands) and 1.5 g/l Osmocote slow release fertilizer (N:P:K = 15:9:11; Scott®, Scotts Miracle-Gro, Marysville, Ohio, USA). Plants were kept for six weeks in a climate room (RH = 70%, light 16h at 20°C, dark 8h at 20°C) and one week prior to the oviposition bioassay plants were placed in the greenhouse.

2.2. Cinnabar moth rearing

Last stage caterpillars of the cinnabar moth were collected from plants of *J. vulgaris* in Meijendel Nature Reserve (52° 7′ 54″ N, 4° 19′ 46″ E, The Netherlands)in July 2009 and were kept in glass tubes until pupation. The pupae were stored in cold a room (4°C) until the next season. In April and May 2010, pupae were taken out of the cold room in three different batches and placed in transparent plastic cages (70 ×70 ×50 cm) under room temperature and natural light. Moths emerged 2-3 weeks later and they were fed for about a week with water and honey before being released in the bioassay. Only healthy and active moths were used.

2.3. Oviposition bioassay

The bioassay was conducted in plastic cylinders (87 cm diameter, ca.1 m high) with a gauze covered top in a greenhouse in the experimental garden of the Institute of Biology in Leiden in May and June 2010 (Fig.S1a,). The cages had a wooden bottom with 20 holes to fix 20 pots with plants so that soil surface was at level with the board (Fig.S1b). Thirty virgin female and 30 virgin male cinnabar moths were released per cage. The plants were watered two or three times during the oviposition bioassay in dishes under the pots without disturbing the cinnabar moths. Cages were rotated every three days to avoid position effects on the oviposition. After ten days, the plants were harvested. The fresh weight was measured for each plant. Digital photographs were taken of all leaves with eggs. The numbers of egg batches per leaf and eggs per egg batch were counted from these photographs (Fig.S1c). In each of the three trials 80 plants were divided over four cages. Twenty different genotypes were placed in one cage according to a random arrangement so each of the forty genotypes was represented by two replicates at each of the three trials.

2.4. PA data

We used the PA data obtained from the experiment described in Chapter 2. PA concentrations were measured by LC-MS/MS in clonal plants that were grown from the same tissue cultures, under identical conditions and consisting of the same genotypes and number of clones, as those used in the cinnabar moth bioassay. We averaged the concentration of each PA across all replicates of each genotype, and these genotypic mean concentrations were used in the analyses presented here, because PA expression is dependent on genotypes under standard growth conditions (Chapter 2). The 37 PAs identified from the *Jacobaea* hybrids could be classified into four types, according to their structural characteristics, biosynthetic pathways and expression patterns: senecionine-, jacobine-, erucifoline- and otonecine-like PAs (Pelser et al, 2005; Chapter 2). We followed this classification in this study. The total concentration of all PAs and the amount of PAs from each structural group were calculated by summing the concentrations of the individual PAs.

2.5. Data analysis

Three variables were used to measure cinnabar moth oviposition preference among the individual plants or hybrid genotype. The variables are: number of eggs per plant; number of egg batches per plant and average egg batch size per plant (the number of eggs per plant/ the number of egg batches per plant). The experiment was not a full three factorial design. Therefore, we first checked the effects of trials and cages by two-way ANOVA and then checked the effect of genotype by one-way ANOVA. We used general linear models to determine whether the three selected indicator variables mentioned above differed among trials and cages. In the three general linear models, trials and cages were defined as the fixed factors; the three indicators were defined as dependent variables, respectively; the fresh weight of the shoot was treated as a covariate (details in Table S1). The ANOVA test results of the models showed that number of eggs per plant and average egg batch size per plant was not affected by trials and cages. However, the number of egg batches per plant seemed to be affected by cages (Table S1). We did ANOVA tests of the number of egg batches per plant against cages trial by trial and found that only one cage in one trial had different number of egg batches from the other cages in the same trial (data not shown).

We also used general linear models to determine whether number of eggs per plant and average egg batch size per plant differed among the plant genotypes. In these general linear models, plant genotypes were defined as the random factor, number of eggs per plant and average egg batch size per plant were defined as dependent variables and fresh weight of the shoot as a covariate. A similar general linear model was conducted to determine whether number of egg bathes per plant differed among the plant genotypes. This model differed from the two models mentioned above in that the independent variable is not the number of egg batches per plant but the residuals of the model with number of egg batches per plant against cages, because the egg batches per plant were different among the cages. Normal distributions and homogenous variances of the general linear models were confirmed by testing the residuals of the models using Shapiro tests and Bartlett tests respectively. The average egg batch size per plant appeared not to be significantly genotype-dependent (Table 1) and was not used in further analyses.

Table 1 ANOVAs of the effects of plant genotypes on the cinnabar moth oviposition preference among 40 F_2 hybrid genotypes from *Jacobaea aquatica* and *Jacobaea vulgaris*

Dependent variables	Independent variables	*df* (k-1)	*Df* (n-k-1)	*F*	*P*
The number of teggs per plant	Hybrid genotype	39	199	1.58	0.02*
	Fresh weight	1	237	4.21	0.04 *
	Error	199			
	Total	240			
The number of egg batches per plant [a]	Hybrid t genotype	39	199	1.99	< 0.001***
	Fresh weight	1	237	6.44	0.01 *
	Error	199			
	Total	240			
Average egg batch size per plant	Hybrid plant genotype	39	199	1.00	0.48
	Fresh weight	1	237	1.28	0.26
	Error	199			
	Total	240			

[a] Residuals of the model with the number of egg batches per plant against cages, because the number of egg batches per plant were different among the cages.
Significance codes: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Linear multiple-regression tests were conducted to check which structural group of PAs affected the oviposition preference of cinnabar moths. The regression was completed in a linear model, in which genotypic mean number of eggs and number of egg batches were selected as independent variables. The genotypic mean total concentrations of each of the four PA groups were used as dependent variables in these models and interactions between the independent variables were included. This model was conducted in R (R Development Core Team, 2009).

One-tailed Pearson or Spearman rank correlation tests were conducted between the genotypic mean number of eggs, number of egg batches and concentrations of individual PAs from the two structural PA groups (jacobine-like PAs and otosenine-like PAs) which were related to the cinnabar moth oviposition preference according to the multiple regression tests (see result section). Since we expected positive correlations only, we used one-sided significance levels in these tests. Whether to conduct a parametric test (Pearson) or a non-parametric (Spearman rank) test depended on the distribution of the PA data. Because we performed multiple tests, the *P*-values of the tests were adjusted in sequential Bonferroni methods,

All analyses except the linear model for multiple regressions (conducted in R) were conducted in SPSS 17.0.

3. Results

3.1 Cinnabar moth oviposition preference among individual plants

The egg batches were always laid on the underside of the leaves. In total 28,323 eggs were found in 1,375 egg batches on 240 plants. On average, each plant received 118 eggs in 5.73 egg batches and on average an egg batch contained 20 eggs. Each female moth on average laid 3.8 egg batches or 78.7 eggs, assuming that all females laid eggs.

The number of egg batches per plant ranged from 0 to 18, with more than 50% of the plants having between four to eight egg batches, less than 10% of the plants having more than 10 egg batches and about 5% of the plants received no egg batches (Figure 1a). The number of eggs per plant ranged from 0 to 534 and more than 50% of the plants had less than 150 eggs (Figure 1b). The number of eggs per plant differed among genotypes and was not different among trials and cages. The number of egg batches differed among genotypes and cages but the average egg batch size per plant did not differ among the plant genotypes (Table 1, supplementary Table 1).

3.2 Relation between cinnabar moth oviposition preference and plant PAs

Multiple-regression showed that two PA groups (jacobine- and otosenine-like PAs) positively correlated to the number of eggs per plant (Table 2). Sum concentration of jacobine-like PAs also positively correlated to the number of egg batches. The other two PA groups (senecionine- and erucifoline-like PAs) were not correlated to the number of eggs or the number of egg batches per plant. There is an interaction between the concentrations of jacobine- and otosenine-like PAs; this interaction was positively correlated to the number of egg batches and the number of eggs per plant (Table 2). This indicated that the effects of jacobine- and otosenine-like PAs on cinnabar moth oviposition preference were positive and the effect may be synergistic.

There are 9 individual PAs in the jacobine group and 7 in the otosenine group. , Strikingly, among the 9 jacobine-like PAs only the tertiary amines were positively correlated to oviposition preference, while there were no significant correlations between the corresponding *N*-oxides and the

Table 2 Results of multiple regressions of the number of eggs and the number of egg batches of the cinnabar moths against the sum concentration of the four structural groups of pyrrolizidine alkaloids (PA, µg/g dw) in the host plants of 40 F_2 hybrid genotypes from *Jacobaea aquatica* and *Jacobaea vulgaris*. For model I (the number of eggs): adjusted $R^2 = 0.37$; $F_{15,24} = 2.53$; $P = 0.020$. For model II (the number of egg batches): adjusted $R^2 = 0.33$; $F_{15,24} = 2.30$; $P = 0.033$.

	Predictors [a]	The number of eggs		The number of egg batches	
		Estimate	*t* value	Estimate	*t* value
	(Intercept)	14.8000	14.376***	5.4690	14.839***
PA groups	snt	0.0042	0.391	0.0003	0.522
	jbt	0.0286	2.186*	0.0013	2.077*
	ert	-0.0397	-0.527	-0.0011	-0.33
	otot	0.4355	2.601*	0.0138	1.779
Two-way interactions	snt:jbt	- 8.06E-06	-0.265	-1.60E-06	-1.137
	snt:ert	2.17E-05	0.174	3.10E-06	0.538
	jbt:ert	-0.0002	-1.575	-5.72E-06	-1.189
	snt:otot	3.80E-05	0.12	-3.03E-06	-0.215
	jbt:otot	0.0011	3.527**	4.87E-05	3.497**
	ert:otot	0.0012	0.443	0.0001	0.825
Three-way interactions	snt:jbt:ert	2.22E-07	0.962	1.24E-08	1.164
	snt:jbt:otot	-8.21E-08	-0.13	-2.86E-08	-1.019
	snt:ert:otot	-3.06E-06	-0.587	1.80E-08	0.075
	jbt:ert:otot	-1.30E-06	-0.335	-1.69E-07	-0.948
Four-way interaction	snt:jbt:ert:otot	-3.24E-10	-0.056	1.39E-10	0.516

[a] Snt, jbt, ert, otot are the sum concentrations of the senecionine-, jacobine- erucifoline- and otosenine-type PAs.
Significance codes: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

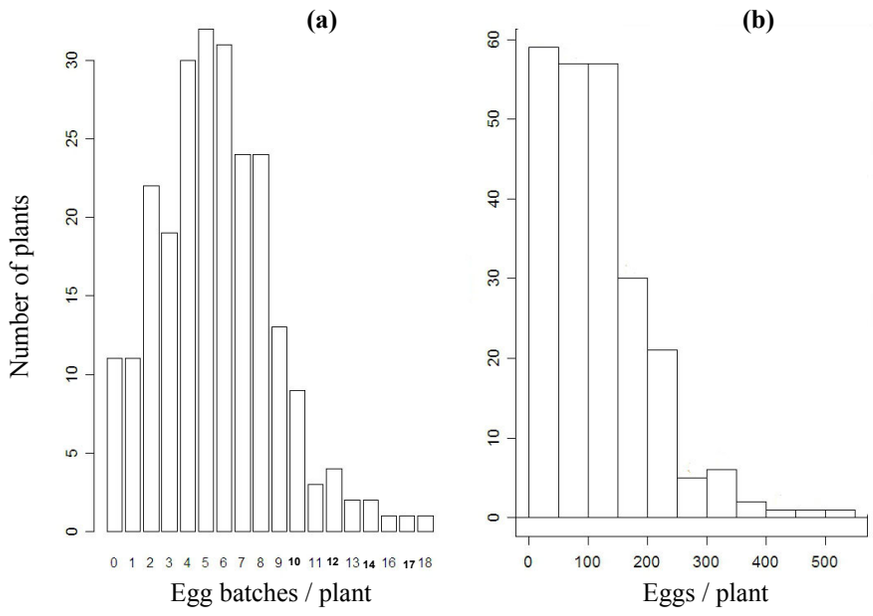


Fig. 1 Frequency distribution of the number of egg batches per plant (a), the number of eggs per plant (b), from 240 plants of 40 F_2 hybrid genotypes of a cross between *Jacobaea vulgaris* and *Jacobaea aquatica*.

Table 3 Results of the one-side Pearson/Spearman correlation tests between the number of eggs and egg batches and concentrations of jacobine- and otosenine-like PAs in the host plants of 40 F_2 hybrid genotypes from *Jacobaea aquatica* and *Jacobaea vulgaris*.

PA group	PA	Code	The number of eggs			The number of egg batchs		
			r/r_s	*P*	Adjusted *P* [a]	r/r_s	*P*	Adjusted *P* [a]
Jacobine-like PAs	jacobine	jb	0.46	**	*	0.43	**	*
	jacoline	jl	0.38	**	ns	0.40	**	ns
	jaconine	jn	0.44	**	*	0.45	**	*
	jacozine	jz	0.39	**	ns	0.48	***	*
	dehydrojaconine	dhjn [b]	0.42	**	*	0.50	***	**
	jacobine *N*-oxide	jbox	0.05	ns	ns	0.13	ns	ns
	jacoline *N*-oxide	jlox	0.08	ns	ns	0.15	ns	ns
	jaconine *N*-oxide	jnox	0.04	ns	ns	0.11	ns	ns
	jacozine *N*-oxide	jzox [c]	0.05	ns	ns	0.04	ns	ns
Otosenine-like PAs [d]	senkirkine	sk	0.25	*	ns	0.26	*	ns
	otosenine	ot	0.24	*	ns	0.12	ns	ns
	onetine	one	0.22	*	ns	0.14	ns	ns
	desacetyldoronine	desdor [c]	0.39	**	ns	0.33	*	ns
	florosenine	fs [c]	0.23	*	ns	0.14	ns	ns
	floridanine	fd [c]	0.28	*	ns	0.18	ns	ns
	doronine	dor [c]	0.19	ns	ns	0.06	ns	ns

[a] *P*-values of the correlation testes were adjusted by sequential Bonferroni method,
[b] This PA was only detected in the tertiary amine form.
[c] Spearman correlation tests were carried out for these PAs without normally distributed concentrations, while Pearson correlation tests were carried out for the other PAs with normal distribution.
[d] Only present as tertiary PAs
Significance codes: ns, $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

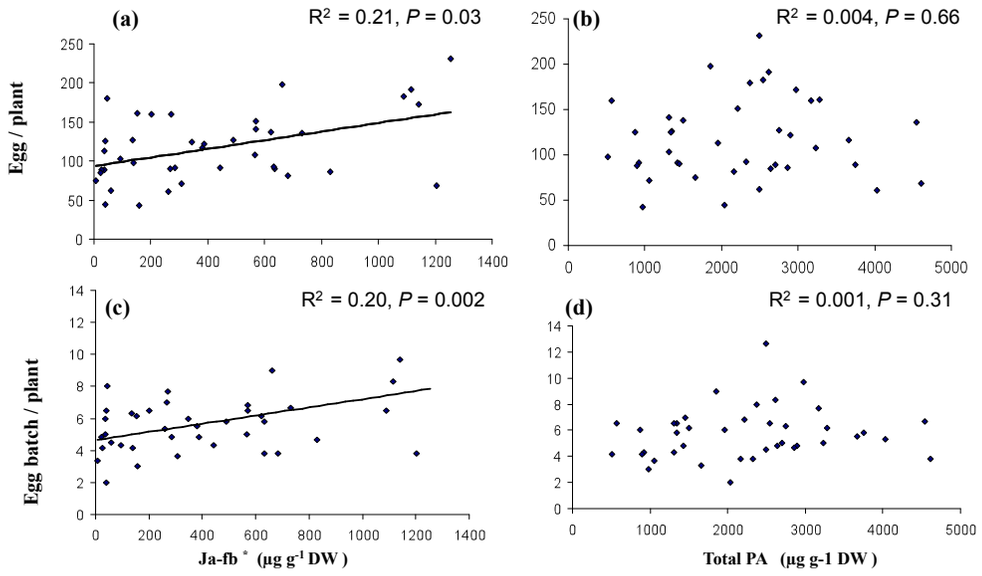


Fig. 2 Scatter graphs of the number of eggs and egg batches per plant against the sum concentration of the 5 jacobine-type tertiary amine PAs (Ja-fb) (a) and total PA (b). Ja-fb are jacobine, jacoline, jaconine, jacozine and dehydrojaconine. Data shown are the genetic mean values of 40 F_2 genotypes of a cross between *Jacobaea vulgaris* and *Jacobaea aquatica*. In both cases: *df* = 38

number of eggs or egg batches (Table 3). The tertiary amines of the five jacobine-like PAs positively correlated to the number of eggs and egg batches. After Bonferroni correction, jacobine, jaconine and dehydrojaconine were significantly correlated to the number of eggs and the number of egg batches. Jacozine was significantly correlated to the number of egg batches only. All otosenine-like PAs (except doronine) positively correlated to the number of eggs and two otosenine-like PAs (senkirkine and desacetyldoronine) positively correlated to the number of egg batches. However, none of the correlations were significant after Bonferroni correction (Table 3). The total concentration of the tertiary amines of jacobine-like PAs explained ca. 20% of the variation of the number of eggs and the number of egg batches among the hybrid genotypes (Figure 2a, c). However, this variation could not be explained by the total PA concentration (Figure 2b, d).

4. Discussion

We demonstrated that the cinnabar moth oviposition preference was affected by the host plant genotype. And we also found that at the genotype level plants with more tertiary amines of jacobine-like PAs and more otosenine-like PAs received more eggs and egg batches (Table 2 and Fig 2a, c). And there were synergistic effects between these two types of PAs. Therefore, those plants with higher levels of these PAs would suffer more damage from cinnabar moths resulting in a lower fitness in environments with abundant cinnabar moths. This indicates that cinnabar moths may potentially act as a selective force on the concentration of jacobine-like tertiary amines. If the amount of this group of PAs is closely correlated to the total PA concentration, like in jacobine-chemotype plants of *J. vulgaris*, the selective force from cinnabar moths may also act on the total amount of PAs. This conclusion agrees with the implication of the high PA concentrations in the invasive ragwort plants compared to ragwort plants in native areas where cinnabars are absent (Joshi and Vrieling, 2005). In previous studies no significant positive correlations between cinnabar moth oviposition preference and PA variation were found in *Jacobaea* plants (Vrieling and de Boer, 1999; Macel et al, 2002). The lack of significant correlations might be due to the fact that the authors did not discriminate the tertiary amine and N-oxide forms of PAs and did not check the relationship between cinnabar moth oviposition preference and individual PAs.

From the view point of herbivores, we may ask why the cinnabar moths preferred host plants with more jacobine-like PAs only. The cinnabar moths used in this study were collected from Meijendel where natural-grown ragwort plants are jacobine chemotypes (Macel et al, 2004) and they may therefore have a preference for plants with jacobine-like PAs. Cinnabar moths from caterpillars collected from a population of Erucifoline chemotype plants may therefore have a preference for erucifoline-like PAs. This hypothesis needs to be tested by conducting oviposition bioassays with cinnabar moths collected from host plants belonging to different chemotypes.

Another interesting question is why significant correlations were observed between the number of cinnabar moth eggs and the jacobine-like tertiary amines but not with the corresponding *N*-oxides of these PAs. A previous study showed that the PA concentration on the leaf surface was marginally correlated to the PA concentration in the leaf tissue and that there were differences in the PA composition on the leaf surface from that of the interior (Vrieling and Derridj, 2003). In *Jacobeae* hybrid plants, the tertiary amines of jacobine-like PAs on surface and the same compounds inside the leaf

were highly correlated and the other PAs did not show such high correlations as these PAs (Cheng et al, unpublished). If female cinnabar moths can only detect PAs on the leaf surface, then the high correlation of tertiary amines of jacobine-like PAs between leaf surface and leaf interior could explain why the cinnabar moths prefer plants with more tertiary amines of jacobine-like PAs in the whole rosette. If cinnabar moths can detect PAs not only on the leaf surface but also inside the leaf, an alternative explanation for the cinnabar moth preference to the plant with more tertiary amines of jacobine-like PAs is that these PAs have a stronger stimulating effect on the cinnabar moth oviposition than other PAs. This could be tested by a cinnabar moth oviposition bioassay with isolated PAs if they are available.

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Supplementary Material

(a)

(b)

(c)

- **Fig.S1**

(a) Cinnabar moth oviposition bioassay conducted in four round plastic cages (*diameter* = 90 cm, height = 1 m) in a greenhouse

(b) Two-level boards with plants but without a cage on. The upper level had 20 holes (ca. 10 cm diameter) to hold 20 plants and on the lower level were dishes for pots. Water could be added to the dishes when the upper level board was moved.

(c) Egg batches from one plant. Purple circles indicate separated egg batches. One leaf had two single eggs but no egg batches, indicated by a blue circle.

- **Table S1** ANOVAs of the effects of trials and cages on cinnabar moth oviposition preference among 40 F_2 hybrid genotypes from *Jacobaea aquatica* and *Jacobaea vulgaris*

Dependent variables	Independent variables	*df* (k-1)	*Df* (n-k)	*F*	*P*
The number of eggs per plant	Trials	2	237	0.15	0.86
	Experimental cages	3	236	1.27	0.28
	Trials × cages	6	233	0.96	0.45
	Error	227			
	Total	240			
The number of egg batches per plant	Trials	2	237	0.91	0.40
	Experimental cages	3	236	2.76	0.04 *
	Trials × cages	6	233	1.01	0.42
	Error	227			
	Total	240			
Average egg batch size per plant	Trials	2	237	0.77	0.46
	Experimental cages	3	236	0.63	0.60
	Trials × cages	6	233	0.50	0.81
	Error	227			
	Total	240			

Significance codes: $^{*}P < 0.05$,

Chapter 5

The Relationship between Structurally Different Pyrrolizidine Alkaloids and Western Flower Thrips Resistance in F_2 Hybrids of *Jacobaea vulgaris* and *Jacobaea aquatica*

Dandan Cheng, Heather Kirk, Klaas Vrieling, Patrick P.J. Mulder, Peter G.L. Klinkhamer

Segregating plant hybrids often have more ecological and molecular variability compared to parental species, and are therefore useful for studying relationships between different traits, and the adaptive significance of trait variation. Hybrid systems have been used to study the relationship between the expression of plant defense compounds and herbivore susceptibility. We conducted a western flower thrips (WFT) bioassay using a hybrid family and investigated the relationship between WFT resistance and pyrrolizidine alkaloid (PA) variation. The hybrid family consisted of two parental (*Jacobaea vulgaris* and *Jacobaea aquatica*) genotypes, two F_1 genotypes, and 94 F_2 hybrid lines. The *J. aquatica* genotype was more susceptible to thrips attack than the *J. vulgaris* genotype, the two F_1 hybrids were as susceptible as *J. aquatica*, and susceptibility to WFT differed among F_2 hybrid lines: 69 F_2 lines were equally susceptible compared to *J. aquatica*, 10 F_2 lines were more susceptible than *J. aquatica* and 15 F_2 lines were as resistant as *J. vulgaris* or were intermediate to the two parental genotypes. Among 37 individual PAs that were derived from four structural groups (senecionine-, jacobine-, erucifoline- and otosenine-like PAs), the *N*-oxides of jacobine, jaconine, and jacoline were negatively correlated with feeding damage caused by WFT, and the tertiary amines of jacobine, jaconine, jacoline, and other PAs did not relate to feeding damage. Total PA concentration was negatively correlated with feeding damage. Among the four PA groups, only the total concentration of the jacobine-like PAs was negatively correlated with feeding damage. Multiple regression tests suggested that jacobine-like PAs play a greater role in WFT resistance than PAs from other structural groups. We found no evidence for synergistic effects of different PAs on WFT resistance. The relationship between PA variation and WFT feeding damage in the *Jacobaea* hybrids suggests a role for PAs in resistance to generalist insects.

Key Words Hybridization, *Jacobaea vulgaris*, *Jacobaea aquatica*, secondary metabolite diversity, chemical defense, *Frankliniella occidentalis*

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1. Introduction

In plants, research into the role of hybridization in the evolution of novel traits and new species is gaining momentum (Barton, 2001; Seehausen, 2004; Abbott et al, 2008). Hybrids have been used increasingly in experimental studies in ecology and evolution in part because interspecific hybrids (specifically segregating generations) often show greater variation in traits compared to parental species. Furthermore, segregating hybrids frequently show greater independence between different traits than the parental species (Hochwender et al, 2000; Lexer et al, 2003; Orians et al, 2010). Interspecific hybrids can have novel patterns of secondary chemical expression or accumulation compared to parental species, and sometimes can be more resistant or susceptible to herbivores than parental species (Rieseberg and Elstrand 1993; Orians 2000; Fritz 1999). This makes hybrids useful for studying the relationship between secondary metabolite variation and herbivores (Hallgren et al, 2003; Leiss et al, 2009).

Hybridization occurs frequently in the *Jacobaea* (syn. *Senecio*, Asteraceae) genus (Vincent, 1996). Members of this genus have been used extensively to study plant-herbivore interactions, which are largely mediated by a diverse group of pyrrolizidine alkaloids (PAs; see reviews by Hartmann, 1999; Macel, 2011). Twenty-six PAs have been reported from 24 *Jacobaea* species (Pelser et al, 2005). PAs are ester alkaloids composed of a necine base (amino alcohol moiety) and an alkyl, or rarely aralkyl, necic acid (Hartmann, 1999). PAs can occur in two forms *in vivo*, the tertiary amine (free base) or the *N*-oxide form (Hartmann et al, 1989; Rizk, 1991; Wiedenfeld et al, 2008; Chapter 3). In *Jacobaea* species, all PAs except for senecivernine are derived from senecionine *N*-oxide, which is synthesized in the roots, transported to the shoots, and diversified into other PA structures (Hartmann and Toppel, 1987). Variation in PA structure and form can lead to variation in the performance of generalist insects and other plant enemies such as nematodes (van Dam et al, 1995; Macel et al, 2005; Dominguez, 2008; Thoden et al, 2009).

Jacobaea vulgaris (tansy ragwort or common ragwort, syn. *Senecio jacobaea*) is native to Europe and west Asia but invasive in North America, Australia and New Zealand. *Jacobaea aquatica* (marsh ragwort, syn. *Senecio aquaticus*) is closely related to, but not a sister species of, *J. vulgaris* (Pelser et al, 2003). Natural hybrids between these species occur in at least one location in The Netherlands (Kirk et al, 2004). The two parental species are attacked by different suites of specialist and generalist herbivores (personal observation). A previous study showed that artificial hybridization between these two species can be used to produce F_2 lines that are in some cases extremely susceptible, and in other cases extremely resistant, to generalist herbivores (Leiss et al, 2009).

Western flower thrips, *Frankliniella occidentalis* (hereafter WFT), is a key insect pest on a wide range of agricultural and horticultural crops globally (Kirk and Terry, 2003). Since this species is highly polyphagous and infests about 200 wild and cultivated host species (Yudin et al, 1986), *F. occidentalis* is often used as a representative generalist herbivore in studies of plant-insect interactions (e.g. Macel et al, 2005; Leiss et al, 2009). Previous studies investigated the effects of PAs on WFT with experiments that used artificial diets (Marcel et al, 2005), or demonstrated the relationship between PAs and WFT resistance in host plants (Macel, 2003; Leiss et al, 2009). These studies showed that PAs are toxic to WFT and play a role in the plant resistance against this insect. However, these authors incorporated only a limited number of PAs in their studies. Some PAs were not easily acquired for experiments. Only the major PAs were quantified in host plants, and PAs were measured without discrimination between the two forms due to technical limitations in analytical methods. The effects of PA variation in host plant on WFT resistance have not yet been tested. This study aimed to overcome the challenges associated with isolating many PA variants for diet studies by measuring WTF resistance in a segregating hybrid family, which is expected to demonstrate great variation in composition and concentration of secondary metabolites such as PAs. Additionally, technological advances now permit the detection of PAs that are present in extremely low concentrations or that demonstrate only slight structural variations compared to other PAs, which allows us to test the relationship between WFT resistance and PA composition using a comprehensive set of PAs *in vivo*.

In this study, we carried out WFT bioassays with an artificial hybrid family including one *J. vulgaris* genotype, one *J. aquatica* genotype, two F_1 offspring, and 94 different F_2 hybrid lines. We measured WFT feeding damage in the shoots of these genotypes, and investigated the relationship between PA variation and susceptibility to attack by WFT in the segregating F_2 generation. We addressed the following questions: 1) Is there variation in WFT resistance among segregating *Jacobaea* hybrids? 2) Is WFT resistance explained by PA concentration and composition, and if so, 3) Do different structural PA variants affect WFT resistance differently? 4) Are there any interactions between the effects of different PAs on WFT resistance?

2. Methods and Material

2.1. Study system and plant growth

Jacobaea vulgaris seeds (collected at Meijendel Nature Reserve, 52° 7′ 54″ N, 4° 19′ 46″ E, The Netherlands) and *J. aquatica* seeds (collected at the Zwanenwater Reserve, 52° 48′ 38″ N, 4° 41′ 7″ E, The Netherlands) were germinated in glass vials. Clones were produced from tissue cultured seedlings, and several clones were subsequently grown in pots in climate rooms under standard conditions (20°C, 70% relative humidity, light: dark 16h: 8h). Potted plants were vernalized at 4°C with the standard light and humidity conditions for approximately 10 weeks to facilitate flowering. Both species are self-incompatible and crosses were performed by rubbing flower heads together (Kirk et al, 2005). Two rayed F_1 offspring were selected from this initial cross, and were reciprocally crossed with each other to produce two sets of F_2 offspring. One F_2 set consisted of 56 individuals and the other consisted of 46 individuals. The parental, F_1 and F_2 individuals were maintained in tissue culture and were cloned to perform experiments using replicate genotypes.

We grew about 6 cloned replicates per F_2 genotype and about 12 cloned replicates per parental and F_1 genotype for the WFT bioassay. In addition we grew the same number of replicates of the genotypes for PA analysis. The PA data for these genotypes were used both to study WFT resistance as described in this paper and for an analysis of patterns of PA profiles in *Jacobaea* hybrid plants that was published elsewhere (Chapter 2). The clones were individually potted in 1.3 liter pots filled with 95% sandy soil (collected from Meijendel), 5% potting soil (Slingerland Potgrond company, Zoeterwoude, The Netherlands) and 1.5 g/l Osmocote slow release fertilizer (N:P:K=15:9:11, Scott®, Scotts Miracle-Gro, Marysville, Ohio, USA). Plants were kept in a climate room under standard conditions described above for six weeks before the bioassay was initiated.

2.2. WFT bioassay

We used 12 replicates of each parental and F_1 genotype, and three to six replicates of each of 94 F_2

hybrid genotypes (six replicates were used for most genotypes, though less than six were used in cases where plants died or were too small compared to other plants of the same genotype). A total of 587 plants were randomly placed in a climate room and grown under standard conditions. About 5870 adult WFT, previously reared on chrysanthemum (*Dendranthema grandiflora*), were released at evenly spaced points in the climate room. During the three week feeding period the plants were watered every two days without wetting or disturbing the leaves. After three weeks, silver damage caused by feeding from WFT on both upper and lower leaf surfaces was visually scored in mm^2 for each leaf, according to the methods developed by Leiss et al (2009). Above ground plant parts (shoots) were harvested just above the root crown and dried for three days in an oven at 50˚C before establishing the dry masses of the shoots.

2.3. PA data acquisition

A Waters Acquity ultra performance liquid chromatographic system coupled to a Waters Quattro Premier XE tandem mass spectrometer (LC-MS/MS) (Waters, Milford, MA, USA) was used for PA analysis. Analysis was performed using a different set of the same tissue culture-derived clonal plants consisting of the same genotypes and number of clones as those used in the WFT bioassay, and grown under identical conditions. The plant shoots were harvested, stored at -80°C and freeze-dried for one week under vacuum with a collector temperature of -55°C. The dried plant material was ground to a fine powder and about 10 mg was extracted with 2% formic acid in a mass to volume ratio of 1:100. Heliotrine (Latoxan, Valence, France) was added as internal standard to the extraction solvent at a concentration of 1 µg/ml. The extract was filtered through a 0.2 µm nylon membrane filter (Acrodisc, Pall Life Sciences, Ann Arbor, MI, USA). An aliquot (25 µl) of the filtered PA extract was diluted with water (975 µl) and injected in to the LC-MS/MS system.

Seventeen individual PA standards were available for this study, representing the major PAs present in the plant extracts (Table 1). Senecionine, seneciphylline, retrorsine and their corresponding *N*-oxides were obtained from Phytolab, Vestenbergsgreuth, Germany; senkirkine was obtained from Phytoplan, Heidelberg, Germany. Riddelliine and its *N*-oxide were obtained as authentic standards from Dr M. Chou (NCTR, Jefferson, AR, USA) and integerrimine was a gift of Dr. J. Trigo (UNICAMP, Campinas, Brasil). Jacobine and erucifoline were isolated from *J. vulgaris* plant material (PRISNA, Leiden, The Netherlands). The identity of the isolated standards was confirmed by ^{1}H-NMR and LC-MS analysis and by comparison with literature data (Logie et al, 1994). Acetyl- seneciphylline was obtained by acetylation of seneciphylline with acetic anhydride and pyridine, according to the procedure described by He et al (2010). Integerrimine *N*-oxide, jacobine *N*-oxide, erucifoline *N*-oxide and acetylseneciphylline *N*-oxide were prepared by *N*-oxidation of the corresponding tertiary amine PAs according to the procedure described by Christie et al (1949) and adapted by Chou et al (2003). The purity of the obtained standards was checked by LC-MS analysis and was at least 90%.

The other PAs listed in Table 1 were tentatively identified on the basis of their retention time, molecular mass and fragmentation pattern and on comparison with PA standards and literature data. The presence of PA *N*-oxides was confirmed by selective reduction to the corresponding tertiary amines according to the method of Joosten et al (2010). All PAs included in this study have been reported before as constituents of *J vulgaris* and/or *J aquatica* (Langel et al, 2011; Hartmann and Witte, 1995, Chapter 3) and no new PAs were identified.

Table 1 Pyrrolizidine alkaloids (PAs) detected in *Jacobaea aquatica*, *Jacobaea vulgaris* and hybrids

Group	PA	Retention time (min)	Precursor mass (m/z)	Fragment mass 1; 2 (m/z)	Collision energy 1; 2 (eV)	Standard PA used for quantification
Senecionine-like PAs (simple senecionine-related derivatives)	senecionine	9.93	336.2	94.0; 120.0	40; 30	senecionine
	senecionine *N*-oxide	6.97	352.2	94.0; 120.0	40; 30	senecionine *N*-oxide
	integerrimine	9.72	336.2	94.0; 120.0	40; 30	integerrimine
	integerrimine *N*-oxide	6.83	352.2	94.0; 120.0	40; 30	integerrimine *N*-oxide
	retrorsine	8.49	352.2	94.0; 120.0	40; 30	retrorsine
	retrorsine *N*-oxide	6.01	368.2	94.0; 120.0	40; 30	retrorsine *N*-oxide
	usaramine	8.29	352.2	94.0; 120.0	40; 30	retrorsine
	usaramine *N*-oxide	5.89	368.2	94.0; 120.0	40; 30	retrorsine *N*-oxide
	riddelliine	7.91	350.2	94.0; 138.0	40; 30	riddelliine
	riddelliine *N*-oxide	5.48	366.2	94.0; 118.0	40; 30	riddelliine *N*-oxide
	seneciphylline	9.16	334.2	94.0; 120.0	40; 30	seneciphylline
	seneciphylline *N*-oxide	6.36	350.2	94.0; 138.0	40; 30	seneciphylline *N*-oxide
	spartioidine	8.96	334.2	120.0; 138.0	30; 30	seneciphylline
	spartioidine *N*-oxide	6.36	350.2	94.0; 138.0	40; 30	seneciphylline *N*-oxide
	acetylseneciphylline	11.80	376.2	120.0; 138.0	30; 30	acetylseneciphylline
	acetylseneciphylline *N*-oxide	8.86	392.2	94.0; 118.0	40; 30	acetylseneciphylline *N*-oxide
	senecivernine	10.09	336.2	94.0; 120.0	40; 30	integerrimine
Jacobine-like PAs (jacobine-related derivatives)	jacobine	7.89	352.2	120.0; 155.0	30; 30	jacobine
	jacobine *N*-oxide	5.49	368.2	120.0; 296.0	30; 25	jacobine *N*-oxide
	jacoline	6.13	370.2	94.0; 138.0	40; 30	jacobine
	jacoline *N*-oxide	4.39	386.2	94.0; 120.0	40; 30	jacobine *N*-oxide
	jaconine	8.75	388.2	94.0; 120.0	40; 30	jacobine
	jaconine *N*-oxide	5.77	404.2	94.0; 138.0	40; 30	jacobine *N*-oxide
	jacozine	7.23	350.2	94.0; 138.0	40; 30	jacobine
	jacozine *N*-oxide	5.11	366.2	94.0; 118.0	40; 30	jacobine *N*-oxide
	dehydrojaconine	7.86	386.2	94.0; 120.0	40; 30	jacobine
Erucifoline-like PAs (erucifoline-related derivatives)	erucifoline	7.56	350.2	94.0; 120.0	40; 30	erucifoline
	erucifoline *N*-oxide	4.80	366.2	94.0; 118.0	40; 30	erucifoline *N*-oxide
	acetylerucifoline	10.18	392.2	94.0; 118.0	40; 30	erucifoline
	acetylerucifoline *N*-oxide	7.17	408.2	94.0; 120.0	40; 30	erucifoline *N*-oxide
Otosenine-like PAs (otosenine-related derivatives)	senkirkine	7.31	366.2	122.0; 168.0	30; 25	senkirkine
	otosenine	5.60	382.2	122.0; 168.0	30; 25	senkirkine
	onetine	4.35	400.2	122.0; 168.0	30; 30	senkirkine
	desacetyldoronine	6.26	418.2	122.0; 168.0	30; 30	senkirkine
	florosenine	8.35	424.2	122.0; 168.0	35; 30	senkirkine
	floridanine	6.79	442.2	122.0; 168.0	30; 30	senkirkine
	doronine	9.01	460.2	122.0; 168.0	30; 30	senkirkine

Data were recorded in multiple monitoring mode (MRM) using two selected precursor ions to product ion transitions per compound. The MS settings are shown in Table 1. For quantification, the sum of the two peak areas obtained for each compound was normalized against the peak area of the internal standard. Quantification was performed against a standard solution (100 µg/l) of the PAs in an extraction of tansy (*Tanacetum vulgare*), a plant known to be free of PAs. The use of a PA standard solution in blank plant extract was considered to be a more reliable approach than quantification against a PA solution in solvent only. This PA standard extraction was injected every 30 samples and the averaged response was used for quantification. For those PAs without standards available, a semi quantitative (indicative) value was obtained by comparison with the most closely related analogue (e.g. an isomer) as indicated in Table 1. Data processing was conducted with Masslynx 4.1 software (Waters, Milford, MA, USA).

PA expression is genetically controlled under standard growth conditions, and PA production is not induced in shoots by aboveground herbivory in *Jacobaea* plants (Vrieling and Bruin, 1987; van Dam et al, 1993; Vrieling et al, 1993). Therefore, we averaged the concentration of each PA across all clones of each genotype and used the genotypic mean concentrations in the analyses presented here. The 37 PAs identified from the *Jacobaea* hybrids could be classified into four types, according to their structural characteristics, biosynthetic pathways and expression pattern: senecionine-like PAs, jacobine-like PAs, erucifoline-like PAs and otonecine-like PAs (Pelser et al, 2005; Chapter 2). We followed this classification in this study (Table 1-2). The total PA concentration as well as the amount for each structural group was calculated by summing the concentrations of the individual PAs.

2.4. Data analysis

We used general linear models to determine whether WFT resistance differed according to plant genotype. Feeding damage (dependent variable) was log- transformed to achieve normality, and plant genotype was defined as the independent variable with plant dry mass as covariate. Normal distributions and homogenous variances were confirmed by testing the residuals of the models using Shapiro tests and Bartlett tests respectively. Two models were set up: in the first model, *J. vulgaris* was used as a reference, and in the second, *J. aquatica* was used as a reference. All other genotypes were compared to the reference in the model. Differences between the hybrid and parental genotypes were evaluated using the regression coefficient matrices of the two models. In each matrix, the estimated coefficient of a hybrid indicated whether it had suffered more or less damage than the reference genotype, and the *P* value showed whether the difference was significant (Crawley, 2005). This is similar to a post-hoc test of an ANOVA model, however such a post-hoc test includes all pair-wise comparisons between groups, and we were only interested in testing for differences between hybrid and parental genotypes. The difference between the two parental genotypes was also tested using the same regression coefficient matrices. WFT resistance of each hybrid genotype was categorized according to these definitions: ND - no difference, leaf damage area of the hybrid was not different from that of both parents; A - additive, damage was intermediate between that of the parents; Ds - susceptible-dominant, damage was similar to that of the susceptible parent; Dr - resistant-dominant, damage was similar to that of the resistant parent, S - susceptible, damage was greater than that of the susceptible parent; R - resistant, damage was less than that of the resistant parent (Table S1).

For correlation tests and principal components analysis, we included only data from F_2 genotypes, since we were interested in using the variation from this segregating generation to search for

Table 2 Pearson/Spearman correlation tests between western flower thrips (WFT) feeding damage and the concentrations of individual pyrrolizidine alkaloids (PAs) in the 94 F_2 hybrid genotypes from *Jacobaea aquatica* and *Jacobaea vulgaris*.

Group	PA	r/r_s	*P*	Adjusted *P* [b]
Senecionine-like PAs	senecionine	-0.247	*	ns
	senecionine *N*-oxide	-0.247	*	ns
	integerrimine	-0.292	**	ns
	integerrimine *N*-oxide	-0.243	*	ns
	retrorsine	-0.201	+	ns
	retrorsine *N*-oxide	-0.104	ns	ns
	usaramine	-0.013	ns	ns
	usaramine *N*-oxide [a]	0.11	ns	ns
	riddelliine [a]	0.217	*	ns
	riddelliine *N*-oxide	-0.109	ns	ns
	seneciphylline	-0.282	**	ns
	seneciphylline *N*-oxide	-0.214	*	ns
	spartioidine	-0.17	+	ns
	spartioidine *N*-oxide	-0.15	ns	ns
	acetylseneciphylline	0.044	ns	ns
	acetylseneciphylline *N*-oxide	0.062	ns	ns
	senecivernine	-0.323	**	+
Jacobine-like PAs	jacobine	-0.281	**	ns
	jacobine *N*-oxide	-0.322	**	+
	jacoline	-0.296	**	ns
	jacoline *N*-oxide	-0.331	**	*
	jaconine	-0.278	**	ns
	jaconine *N*-oxide	-0.325	**	*
	jacozine	-0.141	ns	ns
	jacozine *N*-oxide	-0.057	ns	ns
	dehydrojaconine	-0.102	ns	ns
Erucifoline--like PAs	erucifoline	-0.113	ns	ns
	erucifoline *N*-oxide	-0.081	ns	ns
	acetylerucifoline	-0.209	*	ns
	acetylerucifoline *N*-oxide	-0.195	+	ns
Otosenine--like PAs	senkirkine [a]	0.088	ns	ns
	otosenine	-0.056	ns	ns
	onetine	-0.086	ns	ns
	desacetyldoronine	-0.019	ns	ns
	florosenine [a]	0.252	*	ns
	floridanine [a]	0.234	*	ns
	doronine [a]	0.218	*	ns

[a.] PAs with concentrations that were not normally distributed, for which Spearman correlation tests were carried out, while Pearson correlation tests were carried out for all other PAs.
[b.] *P*-values of the correlation testes were adjusted by Bonferroni method.
Significance codes: [ns] not significant, [+] $P < 0.1$, [*] $P < 0.05$, [**] $P < 0.01$.

underlying relationships between WTF resistance and PA expression. We were not able to test for differences in these relationships between the different generations described in this study because only a limited number of genotypes were included from the parental and F_1 generations. However the parental and F_1 plants provided reference points for WFT resistance comparison. We used log-transformed genotypic mean values of feeding damage and PA concentrations to carry out correlation analyses. Either Spearman (for six minor PAs that did not have normally distributed concentrations) or Pearson correlation tests were carried out to test the relationship between feeding damage and the concentrations of individual PAs, pooled concentrations of each of the four PA groups and total PA(details

in Table 2 and Fig.3).

PAs from within structural groups were closely correlated with each other, and it was therefore not possible to investigate the interactions between them. The PAs from different structural groups, however, were generally expressed independently. The sum concentrations of the PAs from the four groups were not correlated with one another (Chapter 2). We used a multiple-regression model to test for interactions between the effects of different PA structural classes on feeding damage. In this model, feeding damage (represented by log-transformed genotypic mean values) was defined as the dependent variable, and the sum concentrations of each of the four PA structural groups (log-transformed and centered genotypic mean concentrations) were defined as independent variables.

The principal component analysis (PCA) was carried out by using log-transformed genotypic mean concentrations of all individual PAs except the six minor PAs that did not have normally distributed concentrations. Compared to the major PAs these six PAs were present at very low concentrations (on average less than 1% of total PA concentration). Pearson correlation tests were carried out between the first six principle components (PCs) from the PCA and feeding damage. In order to evaluate the contribution of each PA to each PC (in other words the loading), Pearson correlation tests were carried out between individual PAs and the first 3 PCs, since PCs four to six accounted for a low proportion of the total variation and were not correlated with WFT feeding damage. The *P*-values were adjusted using the sequential Bonferroni method when multiple tests were carried out.
All analyses were conducted in R version 2.10.0 (R Development Core Team, 2009)

3. Results

3.1. Variation in feeding damage

Feeding damage was genotype-dependent (*df* = 97,488; *F* = 5.30; *P* < 0.001). Plant mass also had effect on feeding damage (*df* = 1,488; *F* = 18.44; *P* < 0.001). Among the two parental genotypes, *J. aquatica* suffered more feeding damage than *J. vulgaris* (*df* = 1, 22; *t* = 6.18; *P* < 0.001). Both of the F_1 lines were as susceptible as *J. aquatica*. Among the 94 F_2 hybrids, 69 were as susceptible as *J. aquatica*, 10 were more susceptible than *J. aquatica*, 15 showed intermediate resistance, 9 were as resistant as *J. vulgaris*, and none were more resistant than *J. vulgaris* (Fig.1, see statistical details in Table S1).

3.2. Relationship between feeding damage and PA concentration

Correlation tests between feeding damage and individual PAs showed that feeding damage was negatively correlated with the concentrations of the *N*-oxides of two jacobine-like PAs (jaconine and jacoline). Jacobine *N*-oxide concentration was marginally correlated with feeding damage, and the correlations between the free bases of jacobine-like PAs and feeding damage were not significant after correction for multiple testing. No other individual PAs were correlated with feeding damage (Table 2). Total PA concentration was also correlated with feeding damage (Fig.2a). Of the four structural groups of PAs, only the sum concentration of jacobine-like PAs was significantly correlated with feeding damage by WFT (Fig.2c). The sum concentrations of the other three groups were not correlated with feeding damage (see the statistical results for senecionine-and erucifoline-like PAs in Fig2 b,d; for otosenine-like PAs: *df* = 92, *r* = 0.35, *P* = 0.77).

The multiple regression models showed that among the four PA groups only jacobine-like PAs had significant negative effects on feeding. There were no two-way interactions between the groups.

A three-way interaction between senecionine-like, jacobine-like and erucifoline-like PAs and an interaction between the four PA groups were present. However these were only marginally significant (0.05 < *P*< 0.1, Table 3).

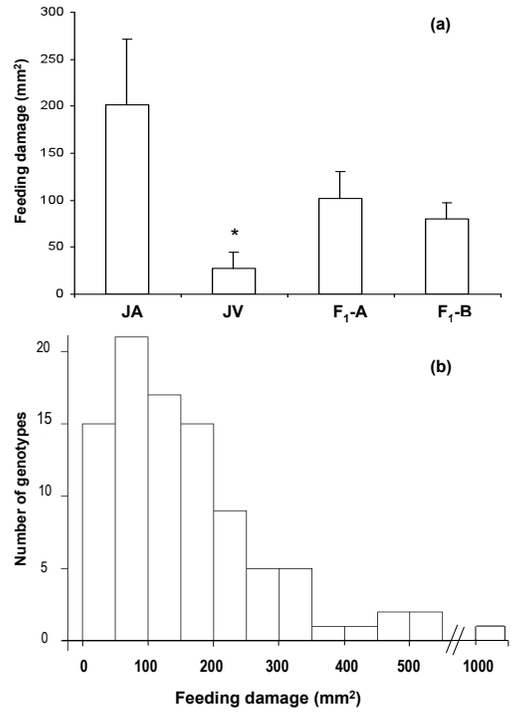

Fig.1 Variation of western flower thrips (WFT) feeding damage (mm^2) in *Jacobaea aquatica, Jacobaea vulgaris*, 2 F_1 and 94 F_2 hybrids. (a) Mean feeding damage for one *J. aquatica* genotype (JA), one *J. vulgaris* genotype (JV), and 2 F_1 (F_1-A and F_1 -B) genotypes. Error bars are standard errors, N = 12. *J. vulgaris* was significantly different from the other genotypes at * P < 0.05. (b) Distribution frequency for genotypic mean WFT feeding damage of 94 F_2 hybrids. N = 3-6 for each genotype. In total, 587 plants were used in WFT bioassay.

3.3. Relationships between feeding damage and PA composition

We used principal component analysis (PCA) to reduce the PA data set to a smaller number of uncorrelated axes. PC1 explained 44%, PC2 explained 19% and PC3 explained 12% of the variation in the data. More than 90% of the total variation was accounted for by the first 6 PCs. Among first 6 PCs, PC1 was negative correlated (*df* = 92, *r* = -0.32, *P* = 0.002) and PC3 was positively correlated with feeding damage (*df* = 92, *r* = -0.23, *P* = 0.04). No other PCs were correlated with feeding damage (data not shown). Correlation tests between each PC and individual PAs concentrations allowed us to identify which PAs were associated with each PC. Jacobine-like PAs (except jacozine and its *N*-oxide) were strongly correlated with PC1, such that individuals with high PC1 scores had high concentrations of jacobine-like PAs. Variation in some senecionine-like, erucifoline-like PAs and otosenine-like PAs contributed strongly to PC3 (individuals with high PC3 scores had low/high concentrations of these PAs; Table S2). A plot of PC1 versus PC2 (Fig.3) shows that F_2 hybrids can roughly be divided into different groups. Feeding damage, indicated by the size of the dots, is not clearly clustered either on the plot of PC1 versus PC2 or the plot of PC_1 versus PC_3 (Fig.3).

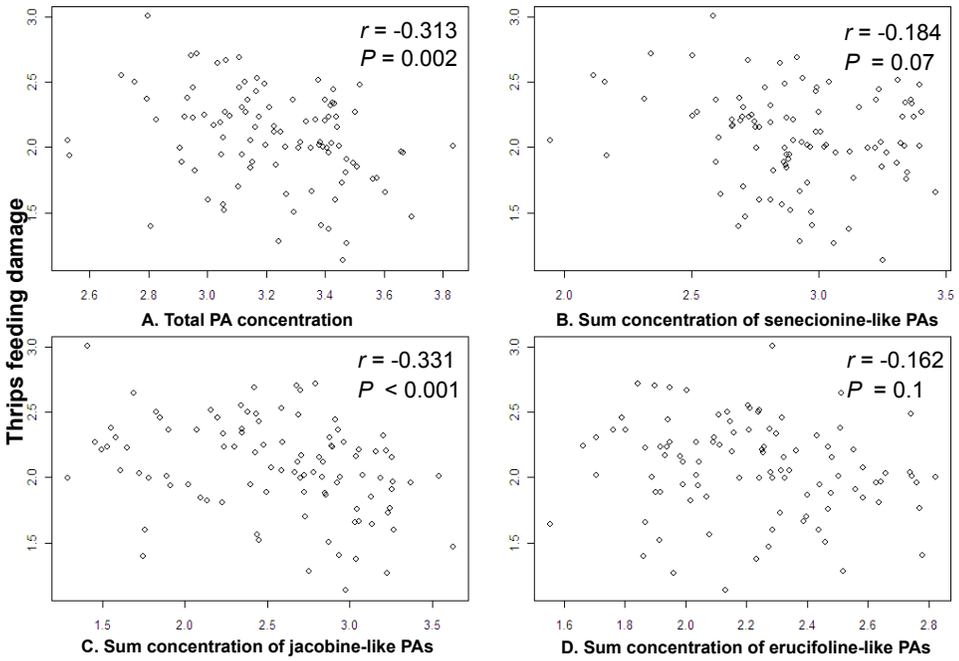

Fig 2. Relationship between feeding damage by western flower thrips (WFT) (mm²) and the concentration of total pyrrolizidine alkaloid (PA), senecionine-like, jacobine-like and erucifoline-like PAs (µg/g dw) of F_2 hybrids of *Jacobaea aquatica* and *Jacobaea vulgaris*. The data for WFT feeding damage and concentrations are the log-transformed genotypic mean values. In each panel the results of the Pearson correlation tests between feeding damage and the PA concentrations are provided; in all cases, *df* = 92.

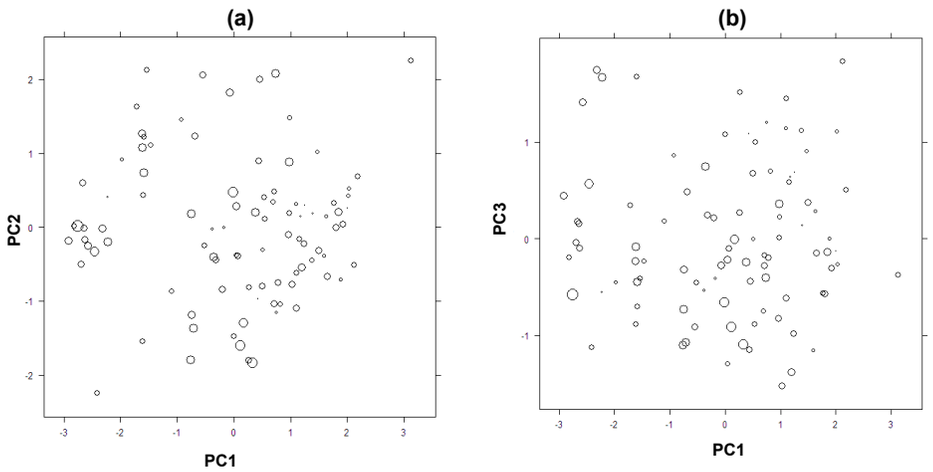

Fig.3 Principle component analysis (PCA) of the pyrrolizidine alkaloid (PA) profiles of F_2 hybrids of *Jacobaea aquatica* and *Jacobaea vulgaris*. PCA was performed on the log-transformed genotypic mean concentrations of all individual PAs excluding six minor PAs that did not have normally distributed concentrations (see Table 2). One dot represents one of 94 F_2 hybrid genotypes. Size of each dot represents mean WFT feeding damage for that genotype. The genotypic mean concentrations are the average value of the three to six replicates from the same genotype.

Table 3 Results of multiple regression of western flower thrips (WFT) feeding damage (mm²) against the sum concentration of four structural groups of pyrrolizidine alkaloids (PAs, µg/g dw) in the 94 F_2 hybrid genotypes from *Jacobaea aquatica* and *Jacobaea vulgaris* (For the regression model: adjusted $R^2 = 0.1655$; *df* = 15, 78; *F* = 2.23; *P* = 0.012)

	Predictors [a]	Estimate	*t* value
PA groups	snt	-0.22	-1.50
	jbt	-0.22	-2.83**
	ert	-0.10	-0.72
	onet	0.06	0.78
Two-way interactions	snt:jbt	-0.43	-1.45
	snt:ert	-0.26	-0.49
	jbt:ert	-0.27	-0.95
	snt:onet	0.05	0.21
	jbt:onet	0.15	0.93
	ert:onet	-0.03	-0.10
Three-way interactions	snt:jbt:ert	1.79	1.89 +
	snt:jbt:onet	0.43	1.07
	snt:ert:onet	-1.19	-1.29
	jbt:ert:onet	1.13	1.63
Four-way interaction	snt:jbt:ert:onet	-3.02	-1.71+

[a] snt, jbt, ert, onet: the sum concentration of senecionine-, jacobine- erucifoline- and otosenine- type PAs, separately.

Significance codes: + P < 0.1, * *P* <0.05, ** *P* < 0.01.

4. Discussion

Segregating hybrids are sometimes used to study correlations and trade-offs between different ecologically important traits in plants, because they exhibit greater variation than parental species, and greater independence between traits (e.g. Orians et al, 2010). We showed that there is high variation in the WFT susceptibility among F_2 hybrids of *J. vulgaris* and *J. aquatica*. Although most F_2 hybrids were as susceptible as or even more susceptible than *J. aquatica* (73% and 11% among all F_2 hybrids respectively), there were still some hybrids with resistance similar to *J. vulgaris* (10%) or intermediate to the two parents (6%). The expression of PAs among the F_2 hybrid generation was highly variable (Chapter 2, and also in Fig 2 and Fig 3), and this variation provided an excellent opportunity to investigate the *in vivo* effects of PA composition on plant resistance to a generalist herbivore.

We demonstrated that concentrations of total PA and jacobine-like PAs were negatively correlated with feeding damage using correlation tests. The multiple regression and PCA also indicated that concentrations of jacobine-like PAs were more closely related to WFT resistance than concentrations of the other PAs. The important role of jacobine-like PAs in WFT resistance of *Jacobaea* plants has also been supported by previous studies. Macel (2003) found that WFT feeding damage was negatively correlated with total PA concentration and with jacobine (both *N*-oxide and free base) concentration in *J. vulgaris* plants. Leiss et al (2009) found that resistant *Jacobaea* hybrids had higher concentrations of jacobine *N*-oxide and jaconine *N*-oxide than susceptible hybrids. To develop a better understanding of the deterrent effects of different PAs on WFT, bioassays should be conducted using pure samples

of different PAs.

Macel et al (2005) tested WFT larval survival on artificial diets containing six individual PAs including senecionine, seneciphylline, retrorsine, senkirkine, heliotrine and monocrotaline, or mixtures of senecionine, seneciphylline, and retrorsine. The experiment indicated that toxic effects of PAs on WFT larva differed among the individual PAs. Furthermore, higher PA concentrations had more potent toxic effects, and no synergistic effects resulted from PA mixtures. These findings support the results of our study, with the caveat that our analysis revealed a potential weak interaction between the different kinds of PAs. However, the interactions were slight ($0.05 < P < 0.1$, Table 3), and it is difficult to interpret interactions between more than two predictors.

PA variation accounted for a relatively low proportion of the variation in feeding damage ($R^2 = 0.17$, Table 3). Therefore, other factors likely play roles in plant susceptibility to WFT. These factors may include plant physical characteristics such as plant size, which was found to be a significant covariate in this study. Total PA concentration and plant size together explained a slightly higher proportion of the total variation ($R^2 = 0.20$). Other secondary metabolites have been reported from these species and their hybrids, including flavonoids, kaempferol glucoside, and chlorogenic acid (Leiss et al, 2009; Kirk et al, 2011), and other phytochemicals such as sesquiterpene lactones may be present but remain unreported. These metabolites may also play a role in resistance to herbivores, individually or in interaction with PAs.

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Supplementary Material

- **Table S1** General linear models of the thrips resistance indicator (thrips feeding damage, mm^2, for the models: $df = 97, 489$; $F = 5.30$; $P < 0.001$)

Genotype	*Jacobaea aquatica* as reference				*Jacobaea vulgaris* as reference				Thrips
	Estimate [b]	Std. Error	*t*	*P*	Estimate	Std. Error	*t*	*P*	Resistance [a]
intercept	2.10	0.13	16.49	<0.001	0.91	0.12	7.50	<0.001	--
The other parent	-1.18	0.18	-6.73	<0.001	1.18	0.18	6.73	<0.001	--
F1-A [c]	-0.25	0.17	-1.46	0.146	0.93	0.17	5.52	<0.001	Ds
F1-B	-0.31	0.18	-1.77	0.078	0.87	0.17	5.07	<0.001	Ds
60127	-0.83	0.21	-3.88	<0.001	0.35	0.21	1.68	0.093	Dr
60129	-0.93	0.21	-4.35	<0.001	0.25	0.21	1.20	0.232	Dr
60152	-0.86	0.27	-3.12	0.002	0.33	0.27	1.20	0.231	Dr
60161	-1.13	0.21	-5.26	<0.001	0.06	0.21	0.27	0.784	Dr
60223	-0.92	0.21	-4.28	<0.001	0.27	0.21	1.28	0.202	Dr
60260	-0.91	0.21	-4.23	<0.001	0.28	0.21	1.32	0.187	Dr
60270	-0.95	0.21	-4.45	<0.001	0.23	0.21	1.10	0.270	Dr
70120	-0.87	0.21	-4.06	<0.001	0.32	0.21	1.50	0.135	Dr
70159	-0.76	0.27	-2.77	0.006	0.42	0.27	1.56	0.120	Dr
60135	0.50	0.21	2.31	0.021	1.68	0.21	7.97	<0.001	A
60145	0.49	0.21	2.29	0.022	1.68	0.21	7.94	<0.001	A
60156	0.56	0.21	2.64	0.009	1.75	0.21	8.29	<0.001	A
60268	0.46	0.21	2.13	0.033	1.64	0.21	7.78	<0.001	A
70202	0.48	0.21	2.27	0.024	1.67	0.21	7.92	<0.001	A
70217	0.84	0.27	3.07	0.002	2.03	0.27	7.45	<0.001	A
60102	-0.24	0.21	-1.10	0.272	0.95	0.21	4.50	<0.001	Ds
60104	-0.07	0.21	-0.34	0.736	1.11	0.21	5.27	<0.001	Ds
60106	-0.19	0.21	-0.87	0.382	1.00	0.21	4.73	<0.001	Ds
60109	0.34	0.21	1.60	0.110	1.53	0.21	7.24	<0.001	Ds
60110	-0.42	0.21	-1.94	0.053	0.77	0.21	3.65	<0.001	Ds
60116	-0.42	0.21	-1.98	0.049	0.76	0.21	3.61	<0.001	Ds
60125	0.10	0.21	0.47	0.636	1.29	0.21	6.10	<0.001	Ds
60137	0.25	0.21	1.18	0.237	1.44	0.21	6.82	<0.001	Ds
60140	0.12	0.21	0.55	0.582	1.30	0.21	6.18	<0.001	Ds
60141	0.03	0.21	0.14	0.885	1.22	0.21	5.76	<0.001	Ds
60146	-0.39	0.21	-1.83	0.068	0.79	0.21	3.76	<0.001	Ds
60157	0.26	0.21	1.20	0.233	1.44	0.21	6.83	<0.001	Ds
60159	-0.18	0.21	-0.83	0.407	1.01	0.21	4.77	<0.001	Ds
60168	-0.32	0.21	-1.50	0.134	0.86	0.21	4.09	<0.001	Ds
60183	0.39	0.21	1.83	0.068	1.58	0.21	7.47	<0.001	Ds
60184	0.12	0.21	0.54	0.586	1.30	0.21	6.17	<0.001	Ds
60185	0.13	0.21	0.60	0.548	1.31	0.21	6.23	<0.001	Ds
60205	-0.23	0.21	-1.10	0.273	0.95	0.21	4.50	<0.001	Ds
60215	-0.27	0.21	-1.25	0.211	0.92	0.21	4.35	<0.001	Ds
60217	0.26	0.21	1.20	0.232	1.44	0.21	6.83	<0.001	Ds
60220	0.32	0.21	1.50	0.134	1.51	0.21	7.14	<0.001	Ds
60229	-0.16	0.21	-0.76	0.446	1.02	0.21	4.84	<0.001	Ds
60230	-0.31	0.27	-1.12	0.264	0.88	0.27	3.22	0.001	Ds

Continue on next page

Genotype	*Jacobaea aquatica* as reference				*Jacobaea vulgaris* as reference				Thrips Resistance
	Estimate [b]	Std. Error	*t*	*P*	Estimate	Std. Error	*t*	*P*	
60232	-0.20	0.21	-0.94	0.349	0.98	0.21	4.66	<0.001	Ds
60248	-0.37	0.21	-1.73	0.084	0.81	0.21	3.86	<0.001	Ds
60249	-0.42	0.27	-1.54	0.124	0.76	0.27	2.80	0.005	Ds
60256	0.08	0.21	0.38	0.707	1.26	0.21	6.00	<0.001	Ds
60259	-0.13	0.21	-0.62	0.533	1.05	0.21	4.98	<0.001	Ds
60261	-0.04	0.21	-0.19	0.849	1.14	0.21	5.42	<0.001	Ds
60262	0.07	0.21	0.31	0.758	1.25	0.21	5.93	<0.001	Ds
60264	0.33	0.21	1.56	0.120	1.52	0.21	7.20	<0.001	Ds
60265	0.38	0.21	1.79	0.073	1.57	0.21	7.44	<0.001	Ds
60267	-0.13	0.21	-0.60	0.548	1.06	0.21	5.01	<0.001	Ds
60269	0.12	0.21	0.56	0.574	1.30	0.21	6.19	<0.001	Ds
60276	0.06	0.21	0.26	0.792	1.24	0.21	5.88	<0.001	Ds
70101	-0.18	0.21	-0.84	0.402	1.00	0.21	4.76	<0.001	Ds
70103	-0.26	0.21	-1.19	0.234	0.93	0.21	4.41	<0.001	Ds
70106	0.20	0.21	0.95	0.344	1.39	0.21	6.58	<0.001	Ds
70107	-0.39	0.21	-1.82	0.069	0.79	0.21	3.77	<0.001	Ds
70108	-0.01	0.21	-0.06	0.951	1.17	0.21	5.55	<0.001	Ds
70109	-0.31	0.21	-1.47	0.143	0.87	0.21	4.13	<0.001	Ds
70110	0.18	0.21	0.84	0.403	1.36	0.21	6.47	<0.001	Ds
70116	0.15	0.21	0.68	0.495	1.33	0.21	6.31	<0.001	Ds
70125	-0.29	0.23	-1.28	0.200	0.89	0.22	3.98	<0.001	Ds
70132	-0.04	0.21	-0.17	0.864	1.15	0.21	5.44	<0.001	Ds
70135	0.11	0.21	0.52	0.602	1.30	0.21	6.15	<0.001	Ds
70138	-0.16	0.21	-0.74	0.462	1.03	0.21	4.87	<0.001	Ds
70140	-0.10	0.21	-0.46	0.646	1.09	0.21	5.15	<0.001	Ds
70143	-0.05	0.21	-0.24	0.811	1.13	0.21	5.37	<0.001	Ds
70146	-0.32	0.21	-1.51	0.131	0.86	0.21	4.08	<0.001	Ds
70149	0.04	0.21	0.21	0.836	1.23	0.21	5.83	<0.001	Ds
70154	0.08	0.23	0.34	0.731	1.26	0.22	5.62	<0.001	Ds
70160	-0.11	0.21	-0.52	0.605	1.07	0.21	5.09	<0.001	Ds
70201	0.10	0.21	0.46	0.649	1.28	0.21	6.08	<0.001	Ds
70203	0.18	0.21	0.83	0.409	1.36	0.21	6.46	<0.001	Ds
70206	-0.20	0.21	-0.95	0.342	0.98	0.21	4.65	<0.001	Ds
70207	-0.22	0.21	-1.02	0.310	0.97	0.21	4.59	<0.001	Ds
70209	-0.16	0.21	-0.76	0.446	1.02	0.21	4.84	<0.001	Ds
70218	0.33	0.21	1.55	0.123	1.52	0.21	7.19	<0.001	Ds
70220	-0.16	0.21	-0.74	0.460	1.03	0.21	4.87	<0.001	Ds
70222	0.00	0.23	-0.01	0.993	1.18	0.22	5.27	<0.001	Ds
70223	-0.04	0.21	-0.21	0.835	1.14	0.21	5.40	<0.001	Ds
70224	-0.34	0.21	-1.57	0.118	0.85	0.21	4.03	<0.001	Ds
70226	0.08	0.21	0.36	0.716	1.26	0.21	5.99	<0.001	Ds
70229	-0.27	0.21	-1.26	0.210	0.92	0.21	4.34	<0.001	Ds
70231	-0.01	0.20	-0.05	0.960	1.17	0.20	5.85	<0.001	Ds
70235	-0.33	0.27	-1.20	0.231	0.85	0.27	3.14	0.002	Ds
70238	-0.24	0.21	-1.14	0.256	0.94	0.21	4.46	<0.001	Ds
70239	0.23	0.21	1.05	0.292	1.41	0.21	6.69	<0.001	Ds
60101	-0.56	0.23	-2.44	0.015	0.63	0.22	2.80	0.005	S
60118	-0.57	0.21	-2.68	0.008	0.61	0.21	2.89	0.004	S
60209	-0.59	0.23	-2.58	0.010	0.60	0.22	2.66	0.008	S
60221	-0.60	0.27	-2.20	0.028	0.58	0.27	2.13	0.033	S
60118	-0.57	0.21	-2.68	0.008	0.61	0.21	2.89	0.004	S
60209	-0.59	0.23	-2.58	0.010	0.60	0.22	2.66	0.008	S
60221	-0.60	0.27	-2.20	0.028	0.58	0.27	2.13	0.033	S
60245	-0.58	0.21	-2.72	0.007	0.60	0.21	2.86	0.004	S
60257	-0.52	0.21	-2.42	0.016	0.67	0.21	3.16	0.002	S
70111	-0.47	0.21	-2.17	0.030	0.72	0.21	3.41	0.001	S
70117	-0.67	0.21	-3.11	0.002	0.52	0.21	2.46	0.014	S
70151	-0.44	0.21	-2.04	0.042	0.75	0.21	3.54	<0.001	S
70158	-0.62	0.21	-2.88	0.004	0.57	0.21	2.69	0.007	S

[a] Resistance patterns: Dr - dominant to resistant parent (9 F_2 genotypes, 9.57% among all F_2 hybrids); A - additive (resistance was intermediate to parents, 6 F_2 genotypes, 6.38%); Ds - dominant to susceptible parent (71 F_2 genotypes, 73.40%); S - more susceptible than both of the parents (10 F_2 genotypes, 10.62%),
[b] The estimated coefficient of a genotype indicates whether it suffered more or less damage than the reference (one of the parents).
[c] F_1-A and F_1-B represent F_1 hybrids; other genotypes represent F_2 hybrids.

- **Table S2** Statistics results of correlation tests between the first three PCs and individual Pyrrolizidine alkaloids (PAs) from PCA in the shoots of 94 F_2 hybrid genotypes of *Jacobaea aquatica, Jacobaea vulgaris* and the hybrids

Group	PAs [a]	*R* [b]			*P* [c]		
		PC1	PC 2	PC 3	PC 1	PC 2	PC 3
Senecionine-like PAs	senecionine	0.03	0.82	0.17	1	<0.001	1
	senecionine *N*-oxide	0.05	0.86	0.25	1	<0.001	1
	integerrimine	0.24	0.73	0.14	1	<0.001	1
	integerrimine *N*-oxide	0.30	0.75	0.26	1	<0.001	1
	retrorsine	0.54	0.14	-0.04	<0.001	1	1
	retrorsine *N*-oxide	-0.06	0.49	0.20	1	<0.001	1
	usaramine	0.39	0.10	-0.12	0.17	1	1
	riddelliine *N*-oxide	0.13	0.09	0.61	1	1	<0.001
	seneciphylline	0.16	0.55	0.60	1	<0.001	<0.001
	seneciphylline *N*-oxide	0.16	0.58	0.70	1	<0.001	<0.001
	spartioidine	0.25	0.00	0.68	1	1	<0.001
	spartioidine *N*-oxide	0.24	-0.03	0.69	1	1	<0.001
	acetylseneciphylline	0.08	0.53	0.01	1	<0.001	1
	acetylseneciphylline *N*-oxide	0.04	0.57	0.24	1	<0.001	1
	senecivernine	0.26	0.48	0.46	1	0.002	0.004
Jacobine-like PAs	jacobine	0.96	-0.10	-0.11	<0.001	1	1
	jacobine *N*-oxide	0.96	-0.09	0.08	<0.001	1	1
	jacoline	0.97	-0.12	-0.10	<0.001	1	1
	jacoline *N*-oxide	0.96	-0.11	0.05	<0.001	1	1
	jaconine	0.96	-0.13	-0.10	<0.001	1	1
	jaconine *N*-oxide	0.96	-0.11	0.08	<0.001	1	1
	jacozine	0.54	0.14	0.15	<0.001	1	1
	jacozine *N*-oxide	-0.03	0.02	0.79	1	1	<0.001
	dehydrojaconine	0.55	0.13	0.19	<0.001	1	1
Erucifoline--like PAs	erucifoline	0.02	0.15	0.26	1	1	1
	erucifoline *N*-oxide	0.00	0.16	0.56	1	1	<0.001
	acetylerucifoline	0.14	-0.04	0.14	1	1	1
	acetylerucifoline *N*-oxide	0.20	0.00	0.30	1	1	1
Otosenine--like PAs	otosenine	0.26	0.74	-0.46	1	0	0.004
	onetine	0.31	0.75	-0.45	1	0	0.008
	desacetyldoronine	0.27	0.73	-0.47	1	0	0.003

[a] excluding six minor PAs that did not show normally distributed concentrations (see details of six minor PAs in Table 2).
[b] The *R* values are the correlation coefficients from the Pearson correlations tests.
[c] The *P*-values were adjusted using the Bonferroni method.

Chapter 6

The role of pyrrolizidine alkaloids in American serpentine leafminer (*Liriomyza trifolii*) resistance in the hybrids of *Jacobaea vulgaris* and *Jacobaea aquatica*

Dandan Cheng, Cilke Hermans, Karin van der Veen - van Wijk, Patrick P.J. Mulder, Klaas Vrieling, Peter G.L. Klinkhamer

Jacobaea species (Asteraceae) contain pyrrolizidine alkaloids (PAs), which are deterring and toxic to generalist herbivores. To investigate the function of the diversity of PAs in *Jacobaea* species, we examined the relationship between PA variation and resistance against a generalist leafminer (American serpentine leafminer, *Liriomyza trifolii*) in an artificial *Jacobaea* hybrid family including one *Jacobaea vulgaris*, one *Jacobaea aquatica*, two F_1, and 90 different F_2 hybrid genotypes.

We conducted a leafminer bioassay with replicated individuals (genotypes) from the *Jacobaea* hybrid family. We measured size of the plants and counted the number of pupae from each plant. For the F_2 hybrids we analyzed whether the number of pupae differed among genotypes and we examined the relationship between the number of pupae and the concentration of 37 individual PAs, the sum concentration of the 4 groups of structurally related PAs and the total PA concentration.

We showed that genotypes differed significantly in the number of pupae. On average 47 pupae per plant were collected from the *Jacobaea vulgaris* parent and 15 pupae per plant from the *J. aquatica* parent. The two F_1 hybrids (15 and 16 pupae / plant, respectively) resembled *J. aquatica*. We found that for the F_2 hybrid genotypes plant size had a strong positive effect on the number of the pupae, while the total PA concentration and that of the major PAs (senecionine-like and jacobine-like PAs) were not correlated to the number of pupae. There was however a trend of decreasing the number of pupae with increasing otosenine-like PA concentrations, while there was a slight increase of the number of pupae with increasing erucifoline-like PA concentrations.

This result of *L. trifolii* differs from a previous study with the same plant genotypes on resistance against a generalist thrips (*Frankliniella occidentalis*), in which jacobine-like PAs were found to be positively related to thrips resistance. This difference indicates that the contribution of plant PAs to herbivore resistance is herbivore species-specific.

Key Words: Secondary metabolites, diversity, pupae survival, generalist herbivores, chemical defense

1. Introduction

Pyrrolizidine alkaloids (PAs) are amongst the most well-known plant defence metabolites. They are ester alkaloids composed of a necine base (an amino dihydroxy moiety) and one or two alkyl necic acids (Hartmann, 1999). The PAs occur in two forms *in vivo*: the tertiary amine (free base) form or the *N*-oxide form (Hartmann et al, 1989; Rizk, 1991; Wiedenfeld et al, 2008). PAs are toxic to mammals (Wiedenfeld and Edgar, 2011). PAs have deterring and toxic effects on generalist insects but stimulate the oviposition and feeding of several specialist insects (see reviews by Boppre, 1986, Hartmann, 1999 and Macel, 2011). *In vitro* experiments with isolated PAs showed that structurally different PAs can have different effects on generalist insects; some are more toxic or deterring than others (Macel et al, 2005). It is generally regarded that the tertiary PAs are more toxic than the corresponding *N*-oxides (van Dam et al, 1995; Macel et al, 2005).

Most *Jacobaea* (syn. *Senecio*, Asteraceae) species contain PAs. *Jacobaea vulgaris* (tansy or common ragwort, syn. *Senecio jacobaea*) is native to Europe and west Asia but invasive in North America, Australia and New Zealand. *Jacobaea aquatica* (marsh ragwort, syn. *Senecio aquaticus*) is closely related to, but not a sister species of, *J. vulgaris* (Pelser et al, 2003). Natural hybrids between these species occur in at least one location in The Netherlands (Kirk et al, 2004). Thirty-seven PAs have been detected from the F_1 and F_2 hybrids of these two species and these PAs could be divided into four groups: senecionine-, jacobine-, erucifoline- and otosenine-like PAs (Chapter 2). The cinnabar moth (*Tyria jacobaeae*) preferred, among the F_2 hybrids, those with a high concentration of jacobine-like PAs (Chapter 4). Western flower thrips (*Frankliniella occidentalis*) on the other hand caused more damage on plants with low concentration of jacobine-like PAs (Chapter 5). No specific role of the other structural types of PAs in the *Jacobaea* hybrids has been revealed so far. To understand the role of PA diversity in plant resistance, we need to know whether other generalist insects are deterred by PAs as well and if so, whether they are deterred by the same or by other structural groups of PAs. To address these questions, we performed bioassays with the American serpentine leafminer (*Liriomyza trifolii*).

Liromyza trifolii is an extremely polyphagous and widespread insect, and it has become an economically important pest in ornamental industry and agriculture (Parrella, 1987; Kang et al, 2009). *Liriomyza trifolii* also has a wide range of weeds and native species as host plants (Stegmaier, 1966; Smith and Hardman, 1986). This leafminer is especially frequent in the Asteraceae. For instance, chrysanthemum was one of the ornamental plants severely damaged by *L. trifolii*. *Senecio glabellus* in Florida (Stegmaier, 1966), *Senecio vulgaris* and *J. vulgaris* in England (Powell, 1981) have been identified as host plants of *L. trifolii*. Furthermore, *L. trifolii* has developed resistance against certain insecticides (Parrella et al, 1984). Therefore, predators and parasites were used as control agents of this insect and resistant lines of crops were selected or developed. Several secondary metabolites have been associated with plant resistance against *L. trifolii*. For example, in specific castor oil plant (*Ricinus communis* L.) lines total phenol concentrations were related to resistance against *L. trifolii* (Anjani et al, 2010). Similarly, trichome-borne acyl sugars from wild tomato *Lycopersicon pennellii* (Hawthorne et al, 1992), cucurbitane glucosides from the cucurbitaceous plant *Momordica charantia* L. (Mekuria et al, 2005; Mekuria et al, 2006), and, phytol, luteolin and various triterpenoids from sweet pepper *Capsicum annuum* (Kashiwagi et al, 2005a; Kashiwagi et al, 2005b) were found to have deterring effects on oviposition and feeding of *L. trifolii*.

We used hybrid plants in this study, because hybrids have several advantages. Interspecific hybrids (specifically segregating generations) often show greater variation in traits compared to parental species. This makes the hybrids useful for studying the relationship between secondary metabolite and herbivores (e.g. Leiss et al, 2009). Interspecific hybrids can have novel patterns of secondary chemical expression or accumulation compared to parental species, and sometimes can be more resistant or susceptible to herbivores than parental species (Rieseberg and Ellstrand, 1993; Fritz, 1999; Orians, 2000; Cheng et al, 2011). Furthermore, segregating hybrids frequently show greater independence between different traits than the parental species (Hochwender et al, 2000; Orians, 2000; Lexer et al, 2003).

We carried out a *L. trifolii* bioassay with an artificial hybrid family including one *J. vulgaris* genotype, one *J. aquatica* genotype, two F_1 and 90 different F_2 hybrids, We determined in an independent set of plants the concentrations of all individual PAs in the shoots of these genotypes (Chapter 2), and investigated the relationship between the PA variation in plants and the plant susceptibility to the leafminer. In this study we address the following questions: 1) Do plant genotypes differ in leafminer resistance? 2) Is leafminer resistance related to the PA variation in the F_2 hybrid genotypes? 3) If so, do different PAs influence resistance to the leafminer differentially? 4) Are there synergistic or antagonistic effects between PAs with respect to leafminer resistance?

2. Methods and Material

2.1 Pant origin and growth

The hybrid family was established from two parental individuals of *J. vulgaris* and *J. aquatica*. The *J. vulgaris* parent was grown from a seed collected at Meijendel Nature Reserve (52° 7′ 54″ N, 4° 19′ 46″ E, The Netherlands), and the *J. aquatica* parent was grown from a seed collected at the Zwanenwater Reserve (52° 48′ 38″ N, 4° 41′ 7″ E, The Netherlands). Both species are self-incompatible. Crosses were performed by rubbing flower heads together. Two F_1 offsprings were selected from this initial cross (*J. aquatica* as mother and *J. vulgaris* as father) and were reciprocally crossed with each other to produce F_2 hybrids. The parental, F_1 and F_2 individuals were maintained in tissue culture and were cloned to perform experiments using these replicated individuals (genotypes).

Plants were propagated by tissue culture and potted in 1.3 liter pots filled with 95% sandy soil (collected from Meijendel), 5% potting soil (Slingerland Potgrond company, Zoeterwoude, The Netherlands) and 1.5 g/l Osmocote slow release fertilizer (N:P:K=15:9:11, Scott®, Scotts Miracle-Gro, Marysville, Ohio, USA). Plants were kept in a climate room (humidity 70%, light 16h at 20°C, dark 8h at 20°C) for six weeks before the bioassay.

2.2. Leafminer origin and rearing

Leafminers were originally collected from several greenhouses in The Netherlands and a stock population was established and kept in climate rooms for more than ten years. Leafminers were reared on "Ultra Light", an extreme leafminer-susceptible cultivar of chrysanthemum (*Dendranthema grandiflora*) in a climate room (humidity 60%, light 16h at 25°C, dark 8h at 25°C). Under this rearing condition, about 14 days are required from egg deposition to the emergence of pupae: egg stage requires 2.5 day developing, three active larva instars require 1.5 days each, and the time spent as a pupa is about 7 days. Pupae used for the leafminer bioassay were collected in the morning after they had fallen out of the leaf and were stored in the cold room (5°C). It took one week to collect enough pupae. Pupae were all taken out of the cold room and put in a climate room (25°C) at the same time to synchronize development. Adult leafminers emerged after 7 days and were kept for one day before the bioassay started.

2.3. Leafminer bioassay

We used 12 clonal replicates of each parental and F_1 genotype, and six replicates for each of the 90 F_2 hybrid genotypes. In total, 588 plants were arranged randomly in a climate room (humidity 70%, light 16h at 25°C, dark 8h at 25°C). The number of leaves and the length of the longest leaf of each plant was measured just before the start of the bioassay. In total 1764 adult leafminers (male: female, 1:1) were released at 49 points (one point per 12 plants, 36 leafminers per point, yielding an average of three leafminers per plant) and were allowed to choose host plants freely after releasing. The adult leafminers were allowed to deposit eggs on the plants for 24 hours and were then collected by using insect aspirators. The plants (free of adult leafminers) were moved to another climate room (humidity 70%, light 16h at 25°C, dark 8h at 25°C) and located randomly. After six days, the above ground parts (shoots) were cut just above the shoot crown and harvested. Shoots were stored individually in plastic bags and these were kept in a climate room (humidity 70%, light 16h at 25°C, dark 8h at 25°C) for a week. Development of pupae was checked and the temperature of the climate room was switched from 25°C to 20°C 3 days after plant harvesting to slow the pupae development. Scoring of pupae for each plant began four days later when nearly all larvae had pupated. About 1% of all larvae were then still alive and had not yet pupated. These larvae were assumed to have survived and pupated if the experiment would have lasted longer. Plants were checked carefully for remaining larvae or pupae on the leaf tissue. The work of pupae scoring was completed within a week after the plant harvesting.

2.4. PA data

A similar set of plants was grown under the same conditions as the plants used for the leafminer bioassay for the collection of PA data. This experiment has been described in detail in Chapter 2. The tissue culture derived plants were from the same genotypes and the same number of clones were used as in the leafminer bioassay. The plants were grown in the climate room as the leafminer bioassay and under identical, herbivore-free conditions. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to determine the PA concentration. Extraction of plant material, PA analysis and the determination of the PA profiles for the set of genotypes, all has been described in Chapter 2.

The concentration of each individual PA was averaged across the clonal replicates of each genotype. These genotypic mean concentrations were used in the analyses presented here, because PA expression is genetically controlled under the conditions used in this experiment. According to their structural characteristics, biosynthetic pathways and expression pattern, the 37 PAs identified from the *Jacobaea* hybrids could be classified into four PA types: senecionine-like, jacobine-like, erucifoline-like and otonecine-like. (Pelser et al, 2005; Chapter 2). In this study, the total PA concentration and the sum concentration for each structural group were calculated by summing the concentrations of the individual PAs within that group (Table 1-2).

2.5. Data analysis

One-way ANOVA was conducted with data (the number of pupae per plant) from F_2 plants to check whether the leafminer resistance differed among the F_2 genotypes. In this ANOVA test, numbers of pupae collected from each individual plant (dependent variable) were log-transformed to achieve equal variance among the genotypes, plant genotype was defined as the independent variable and log-transformed plant size (length of the longest leaf × number of leaf) as covariate. Normal distributions were confirmed by testing the residuals of the models using Shapiro tests.

PAs from within structural groups were highly correlated with each other, and it was therefore not possible to investigate the interactions between them. The PAs from different structural groups, however, were generally expressed independently (Chapter 2). We therefore used a multiple-regression model to test for the effects of the four different PA structural classes and the interactions between them on leafminer resistance. In this model, the number of leafminer pupae (represented by log-transformed genotypic mean values) was defined as the dependent variable, and the sum concentrations of each of the PA structural groups and size of the plants (log-transformed and centered genotypic means) were defined as independent variables. To avoid the collinearity between some independent variables and the interactions, the data of independent variables were centered (Quinn and Keough, 2002).

For completeness we tested the correlations between the number of leafminer pupae and individual PAs' concentrations. To exclude the effect of the plant size, in the correlation tests, we represented leafminer resistance by the residuals of a linear regression with the number of pupae as dependent variable and the plant size as independent variable (the data of both variables are log-transformed genotypic means). Correlations tests were conducted using Pearson or Spearman correlations, depending on whether the PA concentration values were normally distributed or not.
All analyses were conducted in R version 2.10.0 (R Development Core Team, 2009).

3 Results

3.1. Variation of leafminer resistance among individual plants

The number of pupae per individual plant ranged from 0 to 92 and on average from every individual plant 15 leafminer pupae emerged. For the genotype means, the number of pupae ranged from 3.2 to 46.8 and half of the genotypes had no more than 15 pupae per plant (Fig.1). The two parental genotypes differed significantly with regard to the number of the pupae: on average, from *J. vulgaris* 47 pupae emerged while from *J. aquatica* on average only 15 pupae emerged. The number of pupae collected from the two F_1 genotypes was very similar to that from *J. aquatica* (Fig.1a). The F_2 genotypes differed significantly in the number of pupae per plant (ANOVA: $df = 89,449$; $F = 1.99$; $P < 0.001$; Fig.1b). More pupae emerged from larger plants ($df = 1,449$; $F = 29.33$; $P < 0.001$).

3.2. Correlation between leafminer resistance and PA concentrations

The plant size had a positive effect on the number of pupae, and alone explained about 23% of the variation in the number of pupae between genotypes (Fig.2a). The multiple regression model which combined the effect of the plant size and PAs showed that the plant size had a positive effect on the number of pupae. The model also showed a negative trend on the number of the pupae when the concentration of otosenine-like PAs increased. Furthermore, there were four kinds of interactions between the factors which significantly affected the number of the pupae as well. Two of them are interactions between PAs and the other two are those between PAs and plants size. However, it is difficult to explain the interactions. The model indicated that the different PA-types can exert synergistic or antagonistic effects on the number of emerging pupae of *L. trifolii*. In addition the effect of PA concentration depended on the plant size as well (Table 1).

Among the 37 individual PAs, five were negatively related to the number of pupae (two jacobine-like PAs: jacozine and dehydrojaconine; three otosenine-like PAs: otosenine, onetine and desacetyldoronine) and two were positively related to the number of pupae (one senecionine-like PA: riddelliine

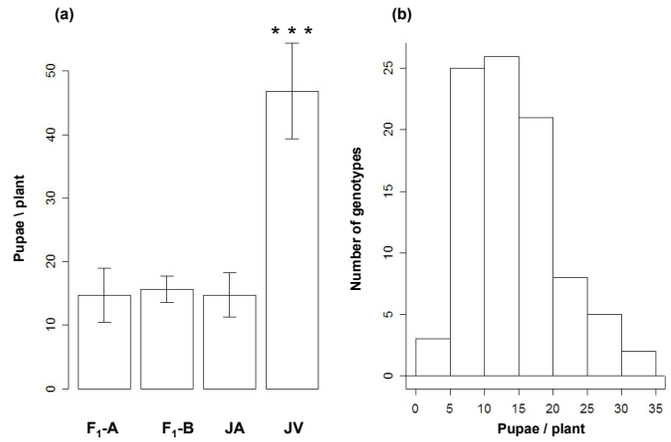

Fig.1 Variation in the number of pupae of the American serpentine leafminer (*Liriomyza trifolii*) collected from J*acobaea aquatica, Jacobaea vulgaris*, and 2 F_1 and 90 F_2 hybrids. (a) Mean number of pupae collected form *J. aquatica* genotype (JA), *J. vulgaris* genotype (JV), and 2 F_1 (F_1-A and F_1-B) genotypes. Each genotype is represented by 12 clonal replicates. Error bars are standard errors. *** $P < 0.001$. (b) Distribution frequency for genotypic mean number of pupae of 90 F_2 hybrids. 3-6 clonal replicates for each genotype. In total, 588 plants were used in the leafminer bioassay.

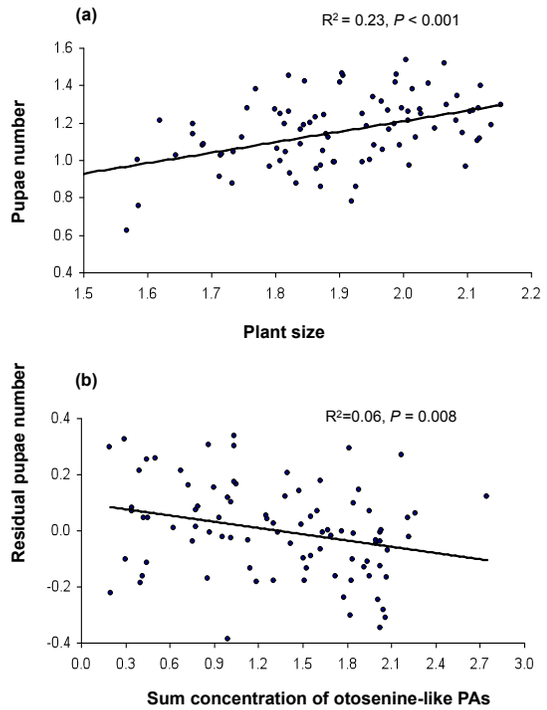

Fig.2 Relationship between American serpentine leafminer (*Liriomyza trifolii*) susceptibility, the plant size and the sum concentration of otosenine-like PAs (µg/g dw) of 90 F_2 hybrid genotypes of *Jacobaea aquatica* and *Jacobaea vulgaris*. Fig.2a: Leafminer susceptibility is represented by the number of pupae per plant. Plant size = length of the longest leaf × number of leaves per plant. Fig.2b: Leafminer susceptibility is represented by the residuals of the number of pupae against the plant size. In all subfigures: data are the log-transformed genotypic mean values.

Table 1 Multiple regression analysis of the number of pupae of American serpentine leafminer (*Liriomyza trifolii*) against the plant size and the sum concentration of four structural groups of pyrrolizidine alkaloids (PAs, µg/g dw) in the 90 F_2 hybrid genotypes from *Jacobaea aquatica* and *Jacobaea vulgaris* (For the regression model: adjusted $R^2 = 0.53$; $df = 31,58$; $F = 4.25$; $P < 0.001$).

	Predictors [a]	Estimate	t value
Factors	Sn-sum	-0.04	-0.58
	Jb-sum	-0.01	-0.24
	Er-sum	0.13	1.92
	Oto-sum	-0.07	-2.24 *
	Plant size	0.56	4.34 ***
Interactions between factors [b]	Sn-sum : Jb-sum	0.49	3.45 **
	Jb–sum : Er-sum	-0.55	-2.41 *
	Er–sum : Plant size	1.06	2.07 *
	Sn-sum : Oto-sum : Plant size	1.65	2.11 *

[a] sn - sum, jb - sum, er - sum, oto - sum: the sum concentration of senecionine-, jacobine- erucifoline- and otosenine-like PAs. Pant size = length of the longest leaf × number of leaves per plant,
[b] The interactions which do not significantly affect the number of pupae are not shown,
* P <0.05, ** P < 0.01, *** P < 0.001

Table 2 Pearson / Spearman correlation tests between the concentrations of pyrrolizidine alkaloids (PAs) and the susceptibility to the American serpentine leafminer (*Liriomyza trifolii*) in 90 F_2 hybrid genotypes of *Jacobaea aquatica* and *Jacobaea vulgaris*. Leafminer susceptibility is represented by the residuals of the number of pupae against the plant size. The data of the number of pupae and the plant size are the genotypic mean values. Size = length of the longest leaf × number of leaves per plant.

Group	PA	r / r_s	P
Senecionine-like PAs	senecionine	-0.084	0.430
	senecionine *N*-oxide	-0.078	0.463
	integerrimine	-0.060	0.576
	integerrimine *N*-oxide	-0.087	0.416
	retrorsine	-0.006	0.953
	retrorsine *N*-oxide	0.165	0.121
	usaramine	-0.069	0.516
	usaramine *N*-oxide [a]	-0.018	0.869
	riddelliine	0.073	0.495
	riddelliine *N*-oxide	0.208	0.049 *
	seneciphylline	-0.120	0.261
	seneciphylline *N*-oxide	-0.073	0.496
	spartioidine	0.054	0.610
	spartioidine *N*-oxide	0.138	0.194
	acetylseneciphylline	-0.147	0.167
	acetylseneciphylline *N*-oxide	-0.183	0.084
	senecivernine	-0.029	0.788
Jacobine-like PAs	jacobine	-0.159	0.135
	jacobine *N*-oxide	-0.144	0.176
	jacoline	-0.155	0.145
	jacoline *N*-oxide	-0.130	0.220
	jaconine	-0.185	0.082
	jaconine *N*-oxide	-0.127	0.232
	jacozine	-0.243	0.021 *
	jacozine *N*-oxide	0.095	0.376
	dehydrojaconine	-0.227	0.031 *
Erucifoline--like PAs	erucifoline	0.122	0.252
	erucifoline *N*-oxide	0.211	0.046 *
	acetylerucifoline	0.175	0.098
	acetylerucifoline *N*-oxide	0.193	0.069
Otosenine--like PAs	senkirkine [a]	-0.148	0.164
	otosenine	-0.226	0.032 *
	onetine	-0.257	0.015 *
	desacetyldoronine	-0.246	0.020 *
	florosenine [a]	-0.067	0.532
	floridanine [a]	-0.090	0.399
	doronine [a]	-0.147	0.167

[a] For PAs with concentrations that were not normally distributed Spearman rank correlation tests were carried out.
Significance codes: ns not significant, *$P < 0.05$, ** $P < 0.01$. Corrected according to the Bonferroni method ($\alpha = 0.05/37 = 0.001$), no correlations are significant.

N-oxide; one erucifoline-like PA: erucifoline *N*-oxide). In fact, all correlation coefficients were quite small and none of the correlations remained significant after Bonferroni correction (Table 2). For the four groups of PAs: the sum concentration of otosenine-like PAs was negatively related to the number of pupae (Fig.2b); erucifoline-like PAs were positively related to the number of pupae (Pearson correlation test: $df = 88$, $r = 0.23$, $P = 0.029$); while no correlation was found for the jacobine-like, senecionine-like PAs or total PA (three Pearson correlation tests: $df = 88$, $P > 0.05$).

4. Discussion

Our study shows that the number of leafminer pupae per plant varied among the plant genotypes. Significantly more pupae developed in larger plants than in smaller ones (Fig.2a). In contrast to our expectation, the effects of major PAs on leafminer resistance were rather weak. Only otosenine-like PAs, a group of PAs that occur often in relatively low concentrations, were slightly negatively correlated to the number of the pupae, which shown by multiple regression and correlation analysis (Fig.2b, Table 2). Surprisingly we even found a weak positive correlation between the number of pupae and the group of erucifoline-like PAs. However, we think it is less likely that erucifoline-like PAs really have positive effects on leafminers. After all, PAs are well known for their negative effects on generalist herbivores (e.g. Macel, 2011). Moreover, erucifoline-like PAs' effects on leafminers were not significant as shown by multiple regression analyses. Multiple regression analyses also showed that there were synergistic and antagonistic effects of PAs and/or the plant size on leafminer pupae (Table 1). However, it is difficult to explain the biological meaning of these interactions.

The sum concentration of the major PA groups (senecionine- and jacobine-like PAs) and total PAs were not related to the number of the pupae. This is a strong indication that the major PAs in *Jacobaea* are not deterring or toxic to the leafminer, at least not in the concentrations present in the plants. The high number of pupae collected from *J. vulgaris* suggests that *J. vulgaris* is in fact the more suitable host plant for *L. trifolii*. Actually, *J. vulgaris* and *S. vulgaris* have been found susceptible to infestation with *L. trifolii* in the field (Powell, 1981). *Senecio glabellus* was identified as one of the host plants of *L. trifolli* in Florida (Stegmaier, 1966). This indicates that *L. trifolii is well* adapted to the PAs present in these plants. For *J. vulgaris* four chemotypes based on their PA profiles were distinguished: 'jacobine chemotype' dominated by jacobine and its derivatives as major PAs; 'erucifoline chemotypes' dominated by erucifoline-like PAs; 'senecionine chemotype' with senecionine-like PAs as dominating PAs; and 'mixed chemotype' with both jacobine- and erucifoline-like PAs as dominating PAs (Witte et al, 1992; Macel et al, 2004). *Senecio vulgaris* has only senecionine-like PAs (Hartmann and Zimmer, 1986; Borstel et al, 1989) and the same is true for *Senecio glabellus* (Ray et al, 1987). None of these species are rich in otosenine-like PAs. This coincides with our finding that the otosenine-like PAs are negatively related to the number of pupae. To confirm that otosenine-like PAs have negative effect on leafminer resistance, it is necessary to perform *in vitro* experiments with pure isolated compounds as some previous work (Hawthorne et al, 1992; Kashiwagi et al, 2005a).

Different to the results for western flower thrips (*Frankniella occidentalis*) obtained with the same genotypes (Chapter 5), PAs do not play an important role in plant resistance against leafminers. This conclusion is in line with a previous study using isolated PAs in artificial diets of generalist herbivores, which showed that six individual PAs (senecionine, retrorsine, seneciphylline, monocrotaline, heliotrine and senkirkine) differed in their toxic or deterrent effect on *Frankliniella occidentalis* (the western flower thrips), *Myzus persicae* (the green peach aphids) and *Locusta migratoria* (grasshopper) while none of the individual PAs deterred feeding by *Spodoptera exigua* (small mottled willow moth) or *Mamestra brassicae* (cabbage moth) (Macel et al, 2005).

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Chapter 7

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 predominately 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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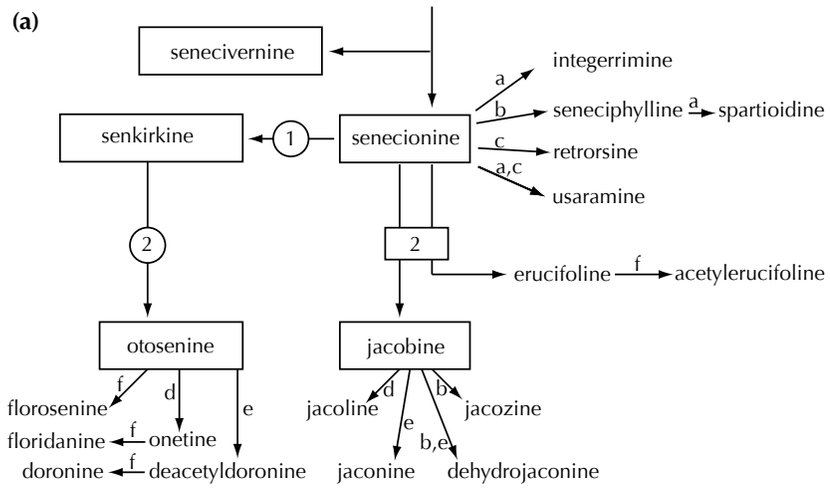
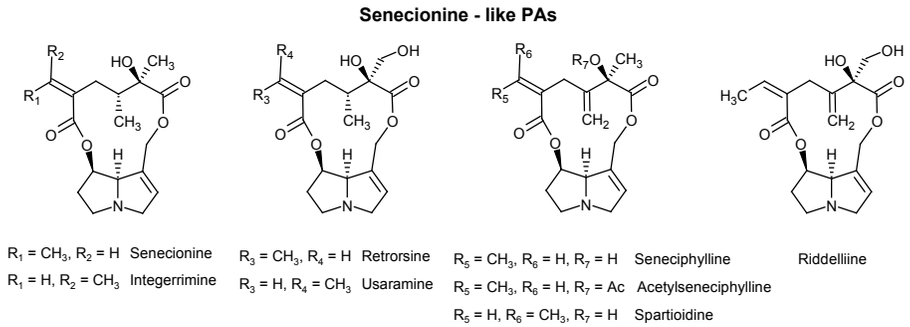


Appendix 1 Putative biosynthetic pathways for diversification of PAs in the *Jacobaea* section.
With the exception of senecivernine, senecionine is the common precursor of all other PAs. Since the substrate specificity of the enzymes involved is not known, two scenarios are illustrated: (a) = senkirkine is assumed to be a common precursor of all otonecine derivatives; (b) = the otonecine derivatives originate independently from the respective retronecine derivatives. Two main reactions exist: conversion of retronecine to otonecine (reaction 1) and site-specific epoxide formation (reaction 2). Further structural diversification requires six simple one-step-reactions marked by letters a–f: a = Z/E-isomerization at C20; b = 13, 19-dehydrogenation; c = site-specific hydroxylations; d = hydrolysis of 15,20-epoxide; e = chlorolysis of 15,20-epoxide; f = site-specific O-acetylations. Adapted from Pelser et al (2005).



Appendix 2 Chemical structures of the pyrrolizidine alkaloids (PAs) found in shoots and roots of *J. aquatica*, *J. vulgaris*, F_1 and F_2 hybrids.

Planten produceren een breed scala van secundaire metabolieten (SMs), zoals glucosiden, saponinen, looistoffen, alkaloïden, etherische oliën en organische zuren (Fraenkel, 1959). Maar veel meer SMs zijn nog niet ontdekt, ramingen van het totale aantal SMS bij planten zijn hoger dan 500.000 (Hadacek, 2002). Er zijn een aantal hypothesen die proberen de SM diversiteit te verklaren en die elkaar niet altijd uitsluiten (zie Hadacek, 2002; Hadacek et al., 2011 en de verwijzingen daar in). Verschillende van deze hypotheses werden naar voren gebracht om de diversiteit van structureel verwante SMs te verklaren in relatie met de verdediging van planten tegen herbivoren. De volgende hypothesen zijn geformuleerd:

1) SMs zijn selectief neutraal. Firn en Jones (2003) ontwikkelden de “Screening Hypothese ‘die ervan uitgaat dat de meeste SMs geen functie hebben voor planten en dat deze noch kosten of baten hebben voor de plant fitness. Toch wordt de SM diversiteit behouden, omdat het de kans vergroot op de productie van nieuwe actievere verbindingen.

2) SM diversiteit is een gevolg van de ‘wapenwedloop’ tussen planten en herbivoren. Nieuw ontwikkelde SMs hebben een sterkere afwerende of toxische effecten op herbivoren. Op hun beurt kunnendeze herbivorenmechanismen evolueren om zich aan te passen aan de nieuwe SMs. Deze continue cyclus heeft geresulteerd in de grote diversiteit van SMs die gevonden kunnen worden in planten (Ehrlich en Raven, 1964). Volgens deze theorie kunnen ook structureel verwantte SMS verschillen in hun effecten op de insect herbivoren en de SMs die het meest recent ontwikkeld zijn, zouden effectiever moeten zijn dan de oudere SMs (Berenbaum en Feeny, 1981; Miller en Feeny, 1983). Het is echter mogelijk dat deze trend niet te zien is in gespecialiseerde herbivoren, die zich sneller kunnen aanpassen aan nieuwe, meer giftige analogen in hun specifieke waardplanten (Cornell en Hawkins, 2003).

3) Planten profiteren van de SM diversiteit vanwege de synergistische effecten tussen de SMs. Mengsels van SMs zouden meer giftig of afstotend zijn dan enkele SMs (Berenbaum et al., 1991; Dyer et al., 2003; Macel et al., 2005).

4) De SM diversiteit wordt in stand gehouden door een selectie van meerdere herbivoren soorten. Iedere herbivoor is gevoelig voor een anders SM en dus zorgt een mengsel van SMs voor een betere verdediging tegen verschillende soorten herbivoren (Mithen et al., 1995; Juenger en Bergelson, 1998; Juenger en Bergelson, 2000; Macel et al., 2005).

Pyrrolizidine alkaloïden (PAs) zijn een klasse van SMs, die constitutief worden gevormd in de planten die ze bevatten en een rol spelen in plant-herbivoor interacties (Hartmann, 1999). Meer dan 400 PAs zijn geïdentificeerd van ca. 6000 soorten angiospermen (Chou and Fu, 2006). Eerdere studies met behulp van in vitro experimenten met gezuiverde verbindingen hebben aangetoond dat de effecten op de plantenetende insecten kunnen verschillen voor structureel verschillende PAs. Ook werden ssynergistische effecten van PAs gevonden (Macel et al., 2005). In dit proefschrift werden de PAs in *Jacobaea* (syn. *Senecio*) soorten gekozen als modelsysteem om de selectieve krachten van insect herbivoren op de PA evolutie te bestuderen. Ik heb in vivo experimenten met een Jacobaea kruising gebruikt in plaats van willekeurig gekozen genotypen van natuurlijke populaties, omdat F2 hybriden

een grote en onafhankelijke variaties in SM expressie en herbivoren resistentie laten zien. De F2 kruising is afkomstig van een kunstmatige kruising tussen Jacobaea aquatica (syn. *Senecio aquaticus*) en *Jacobaea vulgaris* (syn. *Senecio jacobaea*). De kruising omvat ca.100 F_2 hybride genotypen, naast de oudergenortypen van *J. aquatica*, *J. vulgaris* en de twee F_1-hybride genotypen.

De experimentele hoofdstukken van dit proefschrift bestaat uit twee delen: In het eerste deel (hoofdstukken 2 en3) ligt de focus op de PA variatie in de *Jacobaea* hybriden en in het tweede deel (hoofdstukken 4 t/m 6) ligt de nadruk op resistentietegen insectherbivoren en de invloed van PAs op de herbivoren resistentie.

In hoofdstuk 2 heb ik laten zien dat er37 individuele PAs uit de hybride planten geïdentificeerd kunnen worden verdeeld over vier structurele groepen: senecionine-, jacobine-, erucifoline-en otosenine-actige PAs. In de F2 kruising werden transgressie gevonden voor de concnetraties van individuele PAs. Ik vond ook dat binnen elk van de vier groepen de individuele PAs covarieerdenmet betrekking tot de concentratie, maar tussen de verschillende structurele groepen vertoonden de PAs een onafhankelijke segregatie.

In hoofdstuk 3 wordt gevonden dat de planten jacobine-achtige PA aanwezig voor een groter deel als tertiaire aminen aanwezig kunnen zijn dan als N-oxiden. Dit geldt niet voor de andere soorten van PAs. Bovendien, is in individuele planten het aantal tertiaire amines afhankelijk van het plant genotype. De invloed van genetische variatie op het aandeel van de tertiaire amines geeft aan dat in de ecologische en evolutionaire studies over PAs (in het bijzonder van Jacobine-achtige PAs in *Jacobaea* en andere soorten) het belangrijk kan zijn om onderscheid te maken tussen de twee PA vormen. In hoofdstuk 4 wordt aangetoond dat *Tyria jacobaeae* (Jacobsvlinder, een specialistische insect herbivoor) eieren legt op planten op alle F_2-hybride genotypen en er geen PA-afstotende effecten op de ovipositie door *T. jacobaeae* worden waargenomen. Daarmee wordt duidelijk aangegeven dat *T. jacobaeae* goed is aangepast aan de beschikbare PAs. Er werd echter gevonden dat de hybriden met lagere concentraties van tertiaire jacobine-achtige PAs minder belegd werden. In Hoofdstuk 5 wordt gemeld dat schade als gevolg van *Frankliniella occidentalis* (Californische trips, een generalistische herbivoor) afnam met toenemende jacobine-achtige (tertiair amine en N-oxiden) PA-concentratie in de planten. De resultaten van een bioassay met *Liriomyza trifolii* (Amerikaanse serpentijn mineervliegschade, een generalistische insect herbivoor) met planten van de F2 kruising dat het aantal poppen per plant (gecorrigeerd voor omvang van de plant) daalde met toenemende concentratie van otosenine-achtige PAs. De *Jacobaea* hybride familie bleek een goed hulpmiddel om de relatie tussen de PA variatie en herbivoor resisentie te bestuderen, omdat de hybriden grote variatie vertoonden in beide kenmerken. Door middel van drie bioassays, een met een specialist en twee met generalistische insect herbivoren, kon ik zien dat *Jacobaea* hybride genotypen verschilden in de resistentie tegen deze herbivoren en dat deze verschillen gerelateerd waren aan PA variatie in de planten. Niet alle PAs hebeen een gelijk effect op de weerstand tegen plantenetende insecten, en het effect van de PAs is sterk afhankelijk van de getestte herbivoor. In alle drie de bioassays zijn meerdere PAs (ten minste 10 op 37 PAs), betrokken bij de weerstand tegen insecten herbivoren. Deze resultaten voldoen niet aan de voorspellingen die gemaakt kunnen worden op basis van de selectief neutrale theorie, maar ze zijn een ondersteuning van de andere drie hypothesen. De resultaten van de drie bioassays van dit onderzoek en hun belang met betrekking tot de vier hypothesen worden samengevat in Tabel 1. De waarnemingen beschreven in dit proefschrift zijn ondersteunend voor het Generalist-Specialist Dilemma, waarin staat dat de verdediging van kwalitatieve verbindingen in planten generalistische herbivoren zal af te schrikken, maar gespecialiseerde herbivoren zal aan trekken met het gevolg dat deze verschillende selectieve kraschten leiden to een intermediair gehalte aan SM-concentraties (van der Meijden, 1996)

Tabel 1 Overzicht van de relaties tussen pyrrolizidine alkaloïden (PA) concentraties en het gedrag van insecten herbivoren in drie bioassays uitgevoerd met F2 hybriden van een kruising tussen twee Jacobaea soorten. De geselecteerde insecten zijn: De Jacobsvlinder (*Tyria jacobaeae*), Californische trips (*Frankliniella occidentalis*), en de Amerikaanse serpentijn mineervlieg(*Liriomyza trifolii*). Voor de analyse zijn de gemiddelde waarden van de genotypengebruikt. Getallen in de cellen zijn de r- waarden van Pearson correlatie 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}
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	Jacobine-like PAs (tertiary amines)	0.47 **	-0.30 **	0.09 ^{ns}
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	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.
+/-: Test geeft/ geeft geen steun voor de hypothese. ?: The test geeft geen uitsluitsel ovr de desbetreffende hypothese

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I was born in Xishui County, Hubei Province in China on 21, May 1978, which was the 15th day of the 4th month in that year according to traditional Chinese calendar. I started my undergraduate study at Central China Normal University in Wuhan in 1996 and got my B.Sc. in biology in June, 2000. After that, I did my Master of Botany at the same university and graduated in June 2003.

I have been appointed at a permanent position as a teacher and researcher at the Biological Department, School of Environmental Studies, China University of Geosciences (CUG) in Wuhan from July 2003. During my work in CUG, I taught plant biology and ecology.

From when I was a master student, I was involved in a number of projects related to the survey of flora and vegetation in several areas in China with the reference of remote sensing (RS) images.

In June 2007, I was awarded a scholarship by the China Scholarship Council (CSC) of the Ministry of Education for a PhD study at Leiden University. In October 2007 I came to the Netherlands and started my PhD study at the section Plant Ecology (PE) from the Institute of Biology Leiden (IBL).

My research in Leiden resulted in 5 published articles and this thesis.

Dandan Cheng werd geboren in Xishui, Hubei in China, op 21 mei 1978. Dat was de 15e dag van de 4e maand in dat jaar volgens de Chinese kalender. Zij begon haar studie aan de Central China Normal University in Wuhan en behaalde haar bachelordiploma biologie in juni 2000. Daarna volgde zij een masteropleiding in botanie aan dezelfde universiteit en behaalde haar masterdiploma in juni 2003.

Aansluitend werd Dandan benoemd op een permanente positie als docent en onderzoeker op het Biological Department, School of Environmental Studies, China University of Geosciences (CUG) in Wuhan vanaf juli 2003. Tijdens haar werk aan de CUG doceerde zij plantenbiologie en ecologie. Vanaf haar masterstudie was Dandan betrokken bij verschillende projecten waarin vegetatie studies werden gekoppeld aan remote sensing (RS) technieken.

In juni 2007 werd haar een beurs toegekend door de China Scholarship Council (CSC) van het ministerie van onderwijs voor promotieonderzoek aan de Universiteit Leiden. In oktober 2007 kwam Dandan naar Nederland en begon haar promotieonderzoek bij de onderzoek groep Plantenecologie, van het Instituut voor Biologie Leiden (IBL).

Tijdens haar promotieonderzoek bestudeerde Dandan pyrrolizidine alkaloïden (PAs) in hybride *Jacobaea* planten en hun rol in plantenresistentie tegen herbivoren. Haar onderzoek heeft geresulteerd in vijf artikelen in wetenschappelijke tijdschriften en dit proefschrift.

Publication List

- Cheng D, Kirk H, Mulder PPJ, Vrieling K, Klinkhamer PGL (2011). Pyrrolizidine alkaloid variation in shoots and roots of segregating hybrids between *Jacobaea vulgaris* and *Jacobaea aquatica*. New Phytologist 192(4): 1010-1023.
- Cheng D, Kirk H, Vrieling K, Mulder PPJ, Klinkhamer PGL (2011). the relationship between structurally different pyrrolizidine alkaloids and western flower thrips resistance in F_2 hybrids of *Jacobaea vulgaris* and *Jacobaea aquatica*. Journal of Chemical Ecology 37(10): 1071-1080.
- Cheng D, Vrieling K, Klinkhamer PGL (2011). The effect of hybridization on secondary metabolites and herbivore resistance: implications for the evolution of chemical diversity in plants. Phytochemistry Reviews 10(1): 107-117.
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- Kirk H, Cheng D, Choi Y, Vrieling K, Klinkhamer P (2011). Transgressive segregation of primary and secondary metabolites in F_2 hybrids between *Jacobaea aquatica* and *J. vulgaris* Metabolomics. 10.1007/s11306-011-0301-8.

Conference and Workshop

- Dandan Cheng, Eddy van der Meijden, Patrick P.J. Mulder, Klaas Vrieling, Peter G.L. Klinkhamer. Cinnabar moths uses pyrrolizidine alkaloid composition and concentration as a cue for oviposition (poster presentation). The 14th Symposium on Insect-Plant Interactions. 13 – 18, August, 2011, Wageningen, The Netherlands.
- Dandan Cheng, Klaas Vrieling, Patrick P.J. Mulder, Peter G..L. Klinkhamer. Pyrrolizidine alkaloids and the thrips resistance in *Jacobaea* Hybrids (15's oral presentation). The 5th plant-insect interaction workshop.11, November, 2010, Wageningen, The Netherlands
- Dandan Cheng, Klaas Vrieling, Patrick P.J. Mulder, Peter G..L. Klinkhamer. Pyrrolizidine alkaloids and the thrips resistance in *Jacobaea* Hybrids (15's oral presentation). The 21st Netherland Entomological Day. 18, December, 2009, Ede, The Netherlands
- The 3rd workshop of Metabolomics basic and applications to plant sciences. 12 -16, April, 2010, Leiden, The Netherlands

摘要

植物能产生大量的次生代谢物，如苷，皂甙，鞣质，生物碱，挥发油和有机酸等（Fraenkel，1959）。植物次生代谢物的总数估计超过 500,000 (Hadacek, 2002)。植物次生代谢物多样性的进化，也就是其多样性产生和存在的理由，是生物学中众多悬而未决的问题之一，很多理论和假说试图从不同的角度来做出解释(see Hadacek, 2002; Hadacek et al, 2011 and the references there in)。例如，下列 4 种假说是从次生代谢物在植物-植食性动物关系框架内来解释次生代谢物多样性的进化。

选择中性假说：Firn and Jones (2003) 提出“筛选假说”，认为大部分次生代谢物对植物来说没有特别的功能，不会对植物的适合度产生明显的益处或者坏处。但是，次生代谢物多样性的存在增大了植物产生有活性的化合物的可能性，因此被保留下来。

“军备竞赛”假说：这种假说认为次生代谢物多样性是植物和植食性动物之间军备竞赛的结果。在进化的过程中，新产生出来的次生代谢物对昆虫有更强的毒性，但是昆虫经过选择进化后可以适应这些次生代谢物，于是植物又产生新的次生代谢物。这样循环不止的过程最终产生了次生代谢物的高度多样性(Ehrlich and Raven, 1964)。按照这种假说的表述，结构相似的次生代谢物对植食性昆虫的作用可以是不一样的，进化史上新出现的次生代谢物应该比早出现的次生代谢物具有更强的抗虫性 (Berenbaum and Feeny, 1981; Miller and Feeny, 1983)。然而，这样的趋势对于专食性昆虫来说并不明显。因为专食性昆虫很快地适应了在其特异性寄主中产生的新的毒性更强的次生代谢物 (Cornell and Hawkins, 2003)。

协同效应假说：次生代谢物对植食性动物的作用具有协同性，次生代谢物混合物比单独的次生代谢物作用更强，次生代谢物多样性对植物的适合度是有利的(Berenbaum et al, 1991; Dyer et al, 2003; Macel et al, 2005)。

差别效应假说：次生代谢物多样性是植物对来自多种不同植食性动物选择压力的反应。结构相似的次生代谢物对不同的植食性动物的抗性是不一样的。因此，多种次生代谢物的混合物可以抵抗多种植食性动物 (Mithen et al, 1995; Juenger and Bergelson, 1998; Juenger and Bergelson, 2000; Macel et al, 2005)。

吡咯里西啶生物碱(pyrrolizidine alkaloids，PA)是一类介导植物-植食性动物相互作用的代谢物, PA 帮助植物抵御动物的取食(Hartmann, 1999; Macel, 2011)。到目前为止，人们从 6000 多种植物中检测和鉴定到的 PA 有 400 多种(Chou & Fu, 2006)。这 6000 多种植物占了种子植物总数的 3%，分属于 13 个科(Smith & Culvenor, 1981)。超过 95%含有 PA 的植物来自于菊科（泽兰族和千里光族）、豆科（野百合属）、紫草科和兰科（羊耳蒜属）(Hartmann & Witte, 1995)。

对于 PA 多样性的进化，目前还没有满意的解释。用 PA 做体外实验证明结构不同的 PA 对

不同植食性昆虫的毒性是不一样的，而且不同的 PA 对某些昆虫的作用具有协同性(Macel et al, 2005)。在这篇论文里，我选择 *Jacobaea* (syn. *Senecio*) 植物中的 PA 作为研究对象来探讨昆虫是否是 PA 多样性进化的驱动因素。 我研究的植物不是从自然种群中随机挑选的而是来自一个 *Jacobaea* 杂交 F2 家系。因为 F2 代是分离群体，相对来说，在次生代谢物表达和抗虫性方面的变异更大。作为研究系统的杂交家系来源与两个亲本（*Jacobaea aquatica*，*Jacobaea vulgaris*）之间的人工杂交。整个家系包括：2 个亲本、两个 F1 代、100 多个 F2 代个体。

除第 1 章的文献综述，最后一章的总结，本论文用 6 章((Chapter 2-6))报告了相关的实验结果。这 6 章可以分为两个部分：第一部分(Chapter 2-3)重点描述 PA 在所研究植物中的变异；第二部分(Chapter 4-6)描述植物中的 PA 与植物对 3 种不同昆虫的抗性。

第 2 章描述了 PA 测定、分析的结果。在所研究的杂种植物中，一共检测到 37 中 PA，这些 PA 可以分为 4 组：senecionine, jacobine, erucifoline 和 otosenine 组。与预想的一样，在杂种家系中，PA 的表达表现出超亲和独立分离的特征。PA 表达的另一个特征是：在上述 4 个组内，PA 的浓度表现出很高的相关性；而在组间，相关性很低。第 3 章描述的实验结果表明，jacobine-组的 PA 比其他组的 PA 有更多的自由基形式，而其他的 PA 主要以氮氧化物形式存在。而且这两种形式的比例不但跟 PA 相关，也跟植物的基因型相关。

第 3 章描述了 *Jacobaea vulgaris* 的专食性昆虫朱砂夜蛾(cinnabar moth, *Tyria jacobaeae*)在 40 个 F2 代基因型植物上的产卵实验。 实验结果表明，朱砂夜蛾在每个基因型植物上都有产卵，这说明受试植物中的 PA 对朱砂夜蛾没有产卵趋避性，朱砂夜蛾对所有 PA 都有较好的适应性。但是朱砂夜蛾对产卵寄主植物是有选择性的，植物中自由基形式的 jacobine 组 PA 浓度越高，植物叶片背面朱砂夜蛾的卵和卵块就越多。

第 5 章报告了杂交家系中的植物对普适性昆虫西花蓟马(western flower thrips, *Frankliniella occidentalis*)的抗虫性和其含有的 PA 之间的关系。实验结果表明西花蓟马对植物取食造成的伤害面积与植物所含有的 jacobine 组 PA（包括自由基和氮氧化物两种形式）浓度成反比。第 6 章报告杂交家系中的植物对普适性昆虫三裂叶斑潜蝇(American serpentine leafminer, *Liriomyza trifolii*)的抗虫性和其含有的 PA 之间的关系。实验结果表明植物体中寄生的三裂叶斑潜蝇的蛹的数目与植物的 otosenine 组 PA 浓度成反比。第 5-6 章的结果表明 *Jacobaea* 杂交植物的抗虫性与 PA 相关，但不同的 PA 对不同的植食性动物的抗性是不一样的。

综合第 3-6 章的实验结果表明有多种 PA(37 中的 10 种)PA 与 *Jacobaea* 杂交植物的抗虫性相关，而且 PA 间的抗虫作用具有协同性。这与植物代谢物多样性中性选择假说不符，但支持了其他 3 种假说（表 1）。本论文的实验也为普食性-专食性困境理论提供了支持(Generalist-Specialist Dilemma，van der Meijden, 1996)。该理论认为，植物中质量型防御化学物质（如 PA）对普食性昆虫有抗性却吸引专食性昆虫，所以普食性昆虫和专食性昆虫在植物次生代谢物进化中的作用是不一样的。

表 1 *Jacobaea* F2 杂种植物中吡咯里西啶生物碱浓度和 3 种植食性昆虫抗性关系。3 种受试昆虫分别为:朱砂夜蛾(*Tyria jacobaeae*)、西花蓟马(*Frankliniella occidentalis*)、三裂叶斑潜蝇(*Liriomyza trifolii*). 表中数据来源为植物基因型的平均值。表中数字为 Pearson 相关性分析中的 r 值。

昆虫		朱砂夜蛾 (卵块数目, N=40)	西花蓟马 (取食损伤面积, N=98)	三裂叶斑潜蝇（蛹数目/植物个体大小, N=90)
PA 浓度	总 PA（自由基）	0.47 **	-0.32 **	-0.08 ^{ns}
	总 PA（氮氧化物）	0.13 ^{ns}	-0.28 **	-0.05 ^{ns}
	Senecionine 组 PA	-0.06 ^{ns}	-0.18 ^{ns}	-0.16 ^{ns}
	Jacobine 组 PA（自由基）	0.47 **	-0.30 **	0.09 ^{ns}
	Jacobine 组 PA（氮氧化物）	0.13 ^{ns}	-0.34 ***	0.08 ^{ns}
	Erucifoline 组 PA	-0.06 ^{ns}	-0.16 ^{ns}	0.24 *
	Otosenine 组 PA	0.19 ^{ns}	0.03 ^{ns}	-0.33 ***
假说	中性选择假说	-	-	-
	军备竞赛理论	+	+	+
	协同效应假说	+	-	?
	差别效应假说	+	+	+

显著性: ns; * *P*: 0.01-0.05; ** *P*: 0.001-0.01; *** *P*: < 0.001.

+,-: 实验结果支持/不支持某种假说； ?: 实验结果没有明显的证据支持/不支持某种假说。