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

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Chapter 2

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

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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

This chapter was published as:

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: 1010-1023

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).

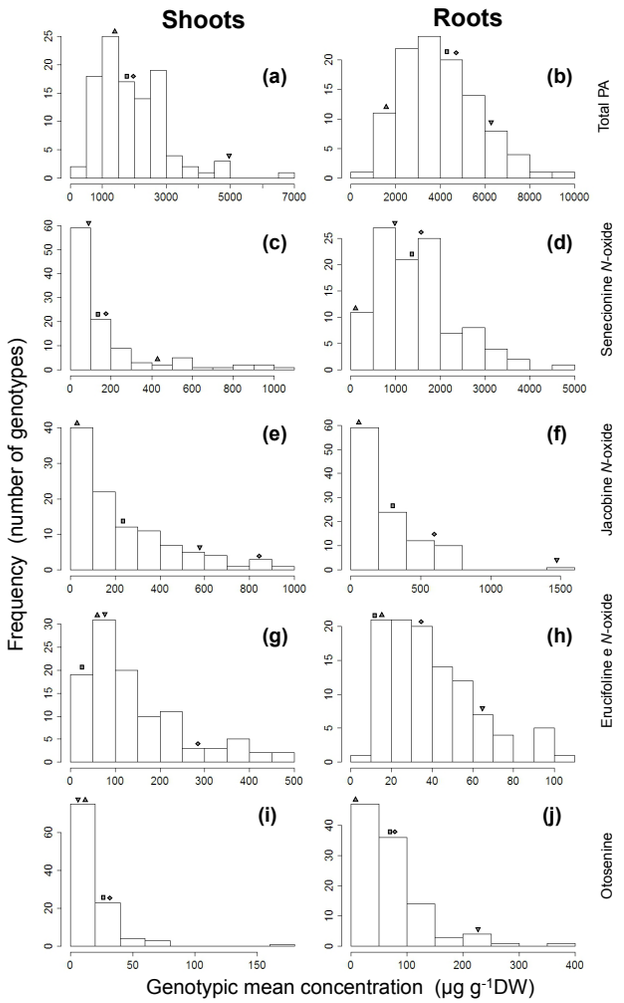

Fig.1 Frequency distribution of genotypic mean concentrations (µg/g DW) of total PA, senecionine *N*-oxide, jacobine *N*-oxide, erucifoline *N*-oxide and otosenine in the shoots and roots of the 102 F_2 hybrid genotypes between *J. aquatica* and *J. vulgaris*. The positions of the symbols above the bars 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).

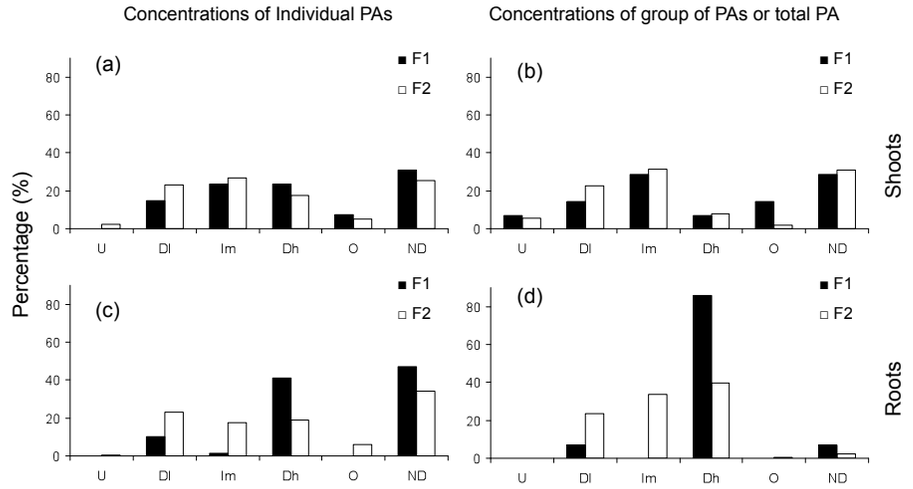

Fig.2 Classification of PA quantitative variation in the shoots and roots of two F_1 and 102 F_2 hybrids relative to the parental genotypes. Hybrid genotypes were classified into six types according to expression of a individual PA, group of PAs or total PA: U (under-expression, significantly less than 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).

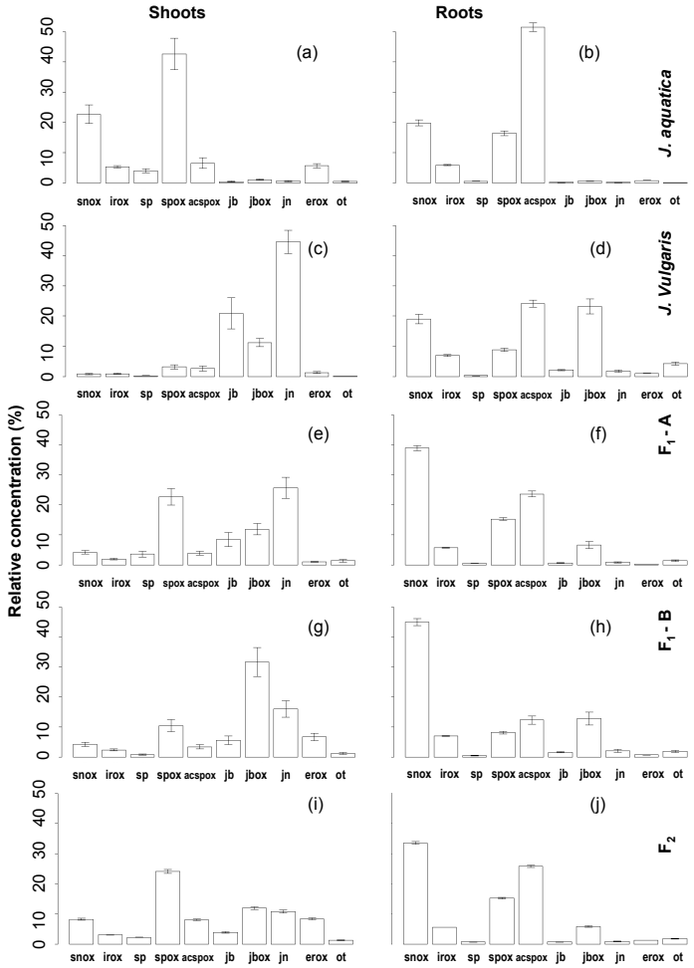

Fig.3 Relative concentrations of major PAs in the shoots and roots of *J. aquatica*, *J. vulgaris*, F_1 and F_2 hybrids. Relative concentrations represent the percentages of total PA concentration in a sample. The PAs shown in the graphs are the 10 PAs with the highest relative concentrations across 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).

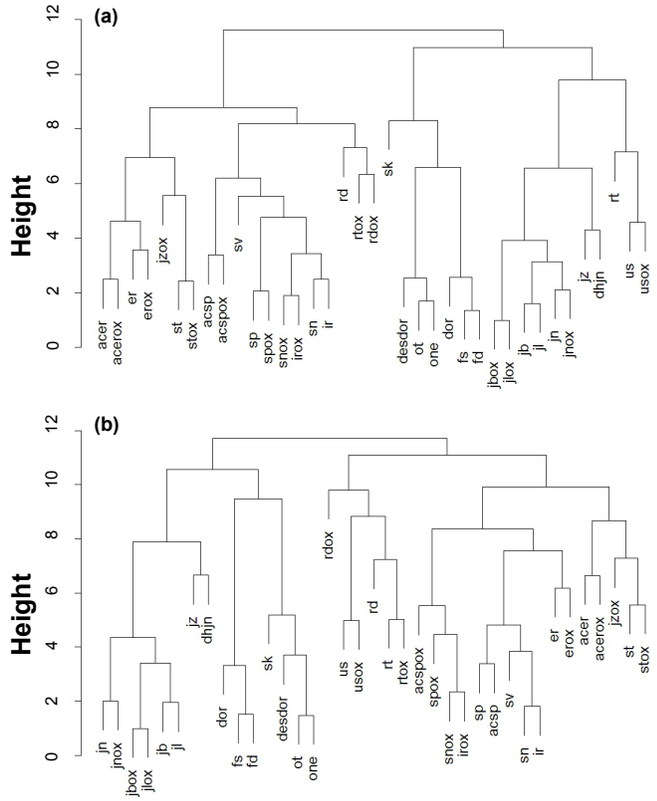

Fig.4 Hierarchical clusters of individual PAs in shoots (a) and roots (b) of *J. aquatica*, *J. vulgaris*, F_1 and F_2 hybrids. The data used in this analysis were the log-transformed absolute concentrations of individual PAs. *J. aquatica*, one genotype, 12 replicates; *J. vulgaris*, one genotype, 12 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.

Acknowledgements

Dandan Cheng thanks the China Scholarship Council (CSC) for financial support. We thank Cilke Hermans, Karin van der Veen-van Wijk, Richard Fens, Meike Klinkhamer and Henk Nell for their technical assistance, Eddy van der Meijden for discussion about experiment design and writing of manuscript, Tom de Jong for the introduction to the statistic software R and Lotte Joosten for the help and suggestions for PA extraction and measurements. We also thank three anonymous referees for their suggestions.

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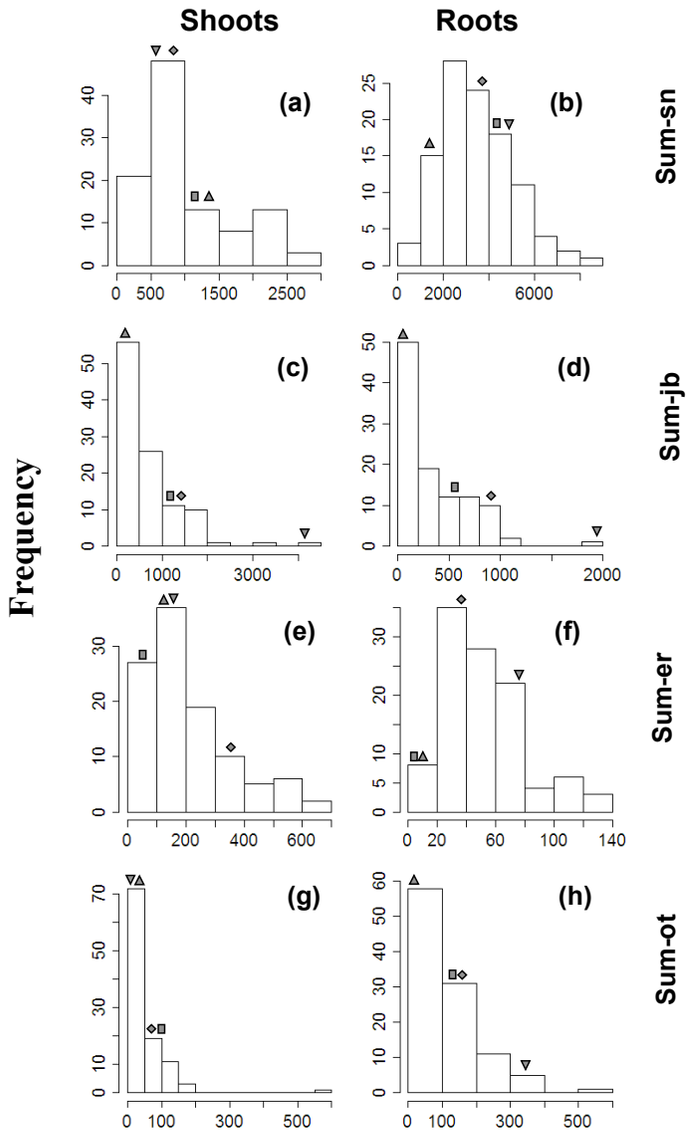
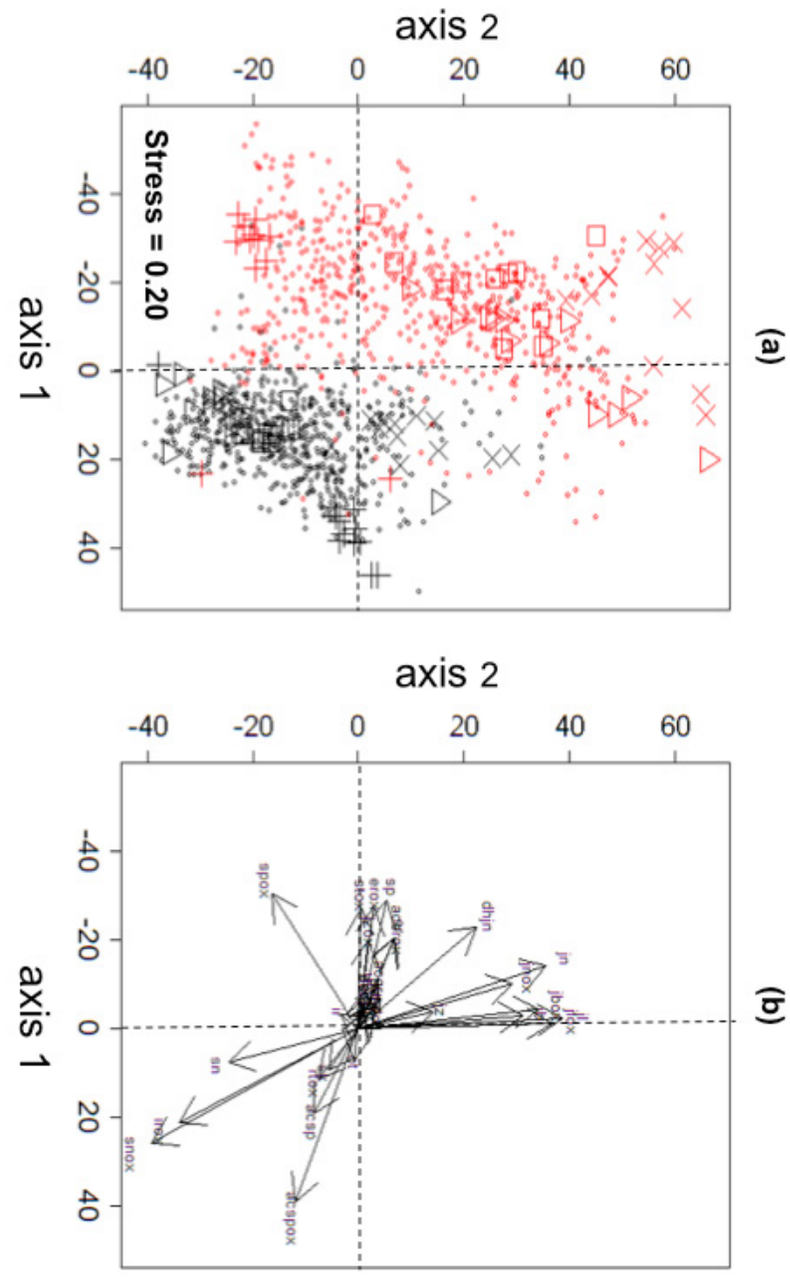
Supplementary material

- **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.