Comparing metabolomes: the chemical consequences of hybridization in plants

Heather Kirk[1], Young Hae Choi[2], Hye Kyong Kim[2], Robert Verpoorte[2] and Ed van der Meijden[1]

[1]Plant Ecology Section, Institute of Biology, Leiden University, PO Box 9502, 2300 RA Leiden, the Netherlands; [2]Division of Pharmacognosy, Section Metabolomics, Institute of Biology, Leiden University, PO Box 9502, 2300 RA Leiden, the Netherlands

Author for correspondence:
Heather Kirk
Tel: +31 71 5275114
Fax: +31 71 5274900
Email: h.e.kirk@umail.leidenuniv.nl

Received: *2 February 2005*
Accepted: *28 February 2005*

Summary

• Hybridization may lead to unique phytochemical expression in plant individuals. Hybrids may express novel combinations or extreme concentrations of secondary metabolites or, in some cases, produce metabolites novel to both parental species.
• Here we test whether there is evidence for extreme metabolite expression or novelty in F_1 hybrids between *Senecio aquaticus* and *Senecio jacobaea*. Hybridization is thought to occur frequently within *Senecio*, and hybridization might facilitate secondary metabolite diversification within this genus.
• Parental species express different quantities of several classes of compounds known to be involved in antiherbivore defence, including pyrrolizidine alkaloids, chlorogenic acid, flavonoids and benzoquinoids. Hybrids demonstrate differential expression of some metabolites, producing lower concentrations of amino acids, and perhaps flavonoids, than either parental species. Despite evidence for quantitative hybrid novelty in this system, NMR profiling did not detect any novel compounds among the plant groups studied.
• Metabolomic profiling is a useful technique for identifying qualitative changes in major metabolites according to plant species and/or genotype, but is less useful for identifying small differences between plant groups, or differences in compounds expressed in low concentrations.

Key words: chemical defence, hybridization, metabolomic profiling, pyrrolizidine alkaloids, secondary metabolites, *Senecio* (Asteraceae).

New Phytologist (2005) **167**: 613–622

© *New Phytologist* (2005) **doi**: 10.1111/j.1469-8137.2005.01448.x

Introduction

Secondary metabolites, defined as compounds that are biosynthetically restricted to a selection of plants (Pichersky & Gang, 2000) and are not used for growth and development, play a significant role in plant survival and fitness. Known to be particularly important for plant interactions with their biotic environment, plant secondary metabolism may mediate interactions with natural enemies including herbivores (Fraenkel, 1959), pathogens (Hol & van Veen, 2002) and competitors (Wardle *et al.*, 1998), and play a role in interactions with pollinators and seed dispersers (Adler, 2000). Furthermore, secondary metabolites are involved in a number of physiological functions including toxic nitrogen storage and transport (alkaloids and peptides), and UV-protectants (flavonoids) (summarized by Wink, 2003).

The surprising diversity of secondary metabolites in plants has been the subject of much debate and experimentation. Despite a number of theories advanced to explain metabolic diversification on an evolutionary time scale (Ehrlich & Raven, 1964; Cornell & Hawkins, 2003), secondary metabolite diversity has been poorly explained from both mechanistic (Pichersky & Gang, 2000) and functional (Wink, 2003) perspectives. However, it has been shown that plant hybridization may have a potentially large role in the evolution of novel secondary metabolites (Rieseberg & Ellstrand, 1993; Orians, 2000), and the formation of new combinations of existing secondary metabolites within plant individuals

(Orians, 2000). Furthermore, previous reports have shown that hybridization can lead to superior resistance to herbivores and pathogens (see review by Fritz, 1999), indicating that innovative expression of secondary metabolites in hybrid individuals may be ecologically adaptive.

If hybridization often leads to the formation of unique metabolites or metabolic profiles, the implications are significant. Historical hybridization events are known to account for 30–70% of modern plant species (Ellstrand *et al.*, 1996 and references therein), indicating that the large-scale diversification of secondary metabolites through hybridization is a possibility. Furthermore, natural hybrid swarms may facilitate the introgression of fitness traits from one species to another (Rieseberg & Wendel, 1993; Rieseberg, 1995). Plant hybridization is therefore thought to have many consequences for evolutionary processes (Arnold, 1997), including the evolution of plant defence.

While hybrid inheritance of individual classes of metabolites (e.g. flavonoids, alkaloids or terpenoids) in many species has been well studied (Rieseberg & Ellstrand, 1993; Orians, 2000), to our knowledge no study has ever examined expression of a wide range of primary and secondary metabolites within hybrid individuals. An integrated understanding of metabolic expression within hybrid individuals may be necessary for accurate estimates of frequency of novel metabolite generation, and a mechanistic understanding of the role of hybridization in phytochemical expression and/or diversification. For instance, a recent review (Orians, 2000) has estimated that the frequency of metabolite novelty resulting from hybridization is between 5 and 20%. This estimate may be biased, as only a limited number of secondary metabolite classes have been screened for novelty in hybrids.

Until recently, large-scale studies of plant metabolites have been limited by the time-consuming and costly nature of available technology. Metabolomics is an emerging field that encompasses the identification and quantification of the suite of metabolites within an organism, tissue or biofluid. Using techniques including nuclear magnetic resonance (NMR) spectroscopy and mass spectroscopy, it is now possible to profile the majority of organic molecules occurring within target samples. Among other uses, metabolomic studies have been applied to the elucidation of biochemical pathways (Weckwerth & Fiehn, 2002; Steuer *et al.*, 2003); to the characterization of ecotypes within species (Ward *et al.*, 2003); and to the identification of metabolomic responses to disease (Griffin, 2003; Viant *et al.*, 2003).

Senecio jacobaea and *Senecio aquaticus* are closely related, nonsister species (Pelser *et al.*, 2003) which form natural hybrid swarms (Kirk *et al.*, 2004). These species demonstrate significant differences in ecological range (Weeda *et al.*, 1991), herbivore susceptibility (personal observation), and resistance to drought and inundation (Kirk *et al.*, 2005). Additionally, *Senecio* species are well known for production of pyrrolizidine alkaloids (PAs), secondary metabolites which are highly toxic to both vertebrate (Cheeke, 1988) and invertebrate herbivores (Frei *et al.*, 1992), and which may influence growth of root-associated fungi (Hol & van Veen, 2002). Hybridization between these species may lead to the formation of novel combinations of PAs in hybrids in the wild (Kirk *et al.*, 2004). However, differences in susceptibility/resistance to specialist and generalist herbivores cannot be completely explained by differences in PA structures, diversity or concentrations (Macel *et al.*, 2002, Nico de Boer, personal communication). Additionally, we have found in herbivore choice tests between *S. jacobaea* and *S. aquaticus* that feeding preference depends on herbivore species (unpublished data). These data lead to the hypothesis that a mosaic of antiherbivore defences play a role in plant–herbivore interactions in these species.

The purpose of this study is twofold. Using a metabolomic profiling approach, we initially aim to identify differential secondary metabolite expression between *S. aquaticus* and *S. jacobaea*, which may be important in ecological interactions. Second, we aim to examine the consequences of hybridization in *Senecio* with regard to a wide range of primary and secondary metabolite expression.

Materials and Methods

Plant material

Both *Senecio jacobaea* L. and *Senecio aquaticus* Hill are biennial to perennial, self-incompatible species. During spring 2003 we transplanted second-year rosettes of *S. jacobaea* and *S. aquaticus* from the Zwanenwater nature reserve in the Netherlands to a glasshouse at Leiden University. On flowering, eight *S. jacobaea* individuals were paired with eight *S. aquaticus* individuals, and pairs were crossed by rubbing flowers together. Seeds were subsequently collected from all plants involved in crosses. We also placed four *S. jacobaea* and four *S. aquaticus* individuals among the plants used for crossing, to control for pollen contamination and/or low levels of selfing in the glasshouse. Control plants did not set any seed, which confirms that seeds harvested from interspecific crosses represent F_1 hybrids. Seeds of *S. jacobaea* and *S. aquaticus* were collected from the Zwanenwater nature reserve.

Seeds of F_1 hybrids, *S. jacobaea* and *S. aquaticus* were germinated, and the following week eight equally sized seedlings from each plant group (*S. jacobaea*, *S. aquaticus* and hybrids) were transplanted to pots (11 cm diameter) filled with dune sand mixed with potting soil (1 : 1). A total of 24 plants were allowed to grow for 8 wk under standard conditions, and all above-ground plant material was harvested for analysis. Plant material was flash-frozen in liquid nitrogen on harvesting and stored at −80°C until extraction.

Extraction of plant material Each sample was freeze-dried. Dry material (100 mg) was transferred to a 10 ml centrifuge tube, and each sample was prepared by addition of 2 ml 50%

methanol-d_4 in buffer (90 mM KH_2PO_4, apparent pH 7) containing 0.05% trimethyl silyl propionic acid sodium salt (TSP, w/v). The mixture was vortexed at room temperature for 30 s, ultrasonicated for 1 min, and centrifuged at 27.9***g*** at 4°C for 20 min. Each NMR sample consisted of 800 µl of the supernatant.

Nuclear magnetic resonance measurements NMR simultaneously detects most organic compounds in a mixed sample, including carbohydrates, organic and fatty acids, amino acids, and most secondary metabolites. Proton (H) NMR functions by detecting protons attached to compounds in a sample, such that one compound is typically represented by multiple signals (protons) in the NMR spectrum. One-dimensional (^{1}H) NMR displays proton signals based on the bonding of each proton to other atoms in compound. Characterization of samples containing hundreds of metabolites, such as those analysed here, can therefore lead to complex spectra with frequent overlap of signals from different compounds. This overlap can lead to poor resolution between compounds if only one-dimensional ^{1}H-NMR methods are employed, as is typical of many metabolomics studies.

To improve the resolution between different signals in the spectra, we screened several kinds of two-dimensional NMR methods to improve resolution. Two-dimensional NMR collects information about the position of each proton in relation to other protons within compounds, to further resolve signals. We found that the J-resolved technique (see Appendix 1 for further details) most improved resolution of the ^{1}H NMR spectra. The two-dimensional spectra were reconverted to a one-dimensional data set (Fig. 1), which produced sharper peaks and better resolution than the true one-dimensional data set. We thus used the projected J-resolved spectra for data analysis.

It is not possible to report the number of compounds appearing in the NMR spectra because multiple peaks represent each compound and identification of all compounds is impractical. However, it is usually estimated that NMR detects a range of 50–100 of the most highly accumulated plant compounds. In our experience, metabolites occurring in concentrations less than ≈ 1 µmol mg^{-1} are generally not detectable using the methods described here. Also, the sensitivity of the analysis depends on the nature of target compounds. Some areas of the spectra, such as the region where many carbohydrates are found (Fig. 1), contain many signals, and metabolites occurring in this region may be difficult to detect if present in low concentrations. Conversely, relatively few signals occur in the phenolic region of the spectra, and even weak signals may be detected here.

Identification of compounds requires further NMR analyses, described in detail in Appendix 2. Briefly, the position, intensity and splitting pattern (number of peaks representing a signal) yield information about the identities of atoms and bonds surrounding each proton. For example, protons attached to a phenolic carbon atom appear in the phenolic region of the spectrum (Fig. 1). Elucidation of structures from a complex mixture therefore requires expert knowledge of plant phytochemistry and NMR spectral patterns.

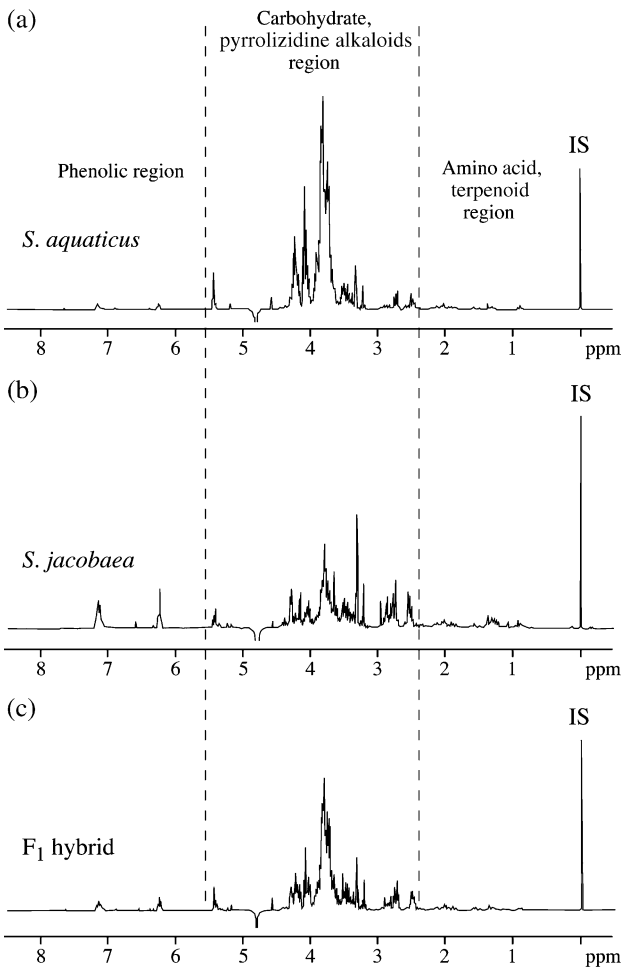


Fig. 1 ^{1}H-NMR spectra for methanol–water extracts of individual samples of *Senecio aquaticus*, *Senecio jacobaea* and an F_1 hybrid of *S. aquaticus* and *S. jacobaea*. IS, internal standard TSP.

Data analysis

Initial data handling The J-resolved projection spectra were automatically exported as a spreadsheet using AMIX software (ver. 3.7, Bruker Biospin). The spectra, which were measured in chemical shift (δ; ppm), were divided (bucketed) into bins of equal width (0.04 ppm), and the peaks represented within each bin were integrated (the area under the spectra was calculated). Integrated signals were scaled (standardized) to the internal standard TSP (intensity = 1000), so that peak intensities represent intensity relative to the internal standard. Integrated and standardized signals were used for all further quantitative analysis of the data. The region from δ −0.4 to

10.0 (Fig. 1) was included in the analysis, and the region from δ 4.7 to 5.0 was excluded from the analysis because the water signal masked all other signals in this region. The region including citric acid, malic acid and succinic acid from δ 2.8 to 2.5 was bucketed by 0.1 ppm because chemical shift of these compounds can differ slightly according to concentration.

We compared the similarity of all pairs of individuals by matching two-dimensional J-resolved spectra using AMIX. The program calculates the percentage of signals that overlap between samples, based on the presence and absence of signals, rather than quantity.

Principal component analysis Principal component analyses (PCA) were performed with the SIMCA-P software (ver. 10.0, Umetrics, Umeå, Sweden). Principal component analysis is an unsupervised clustering method requiring no knowledge of the data set, and acts to reduce the dimensionality of multivariate data while preserving most of the variance within it (Goodacre *et al.*, 2000). The principal components can be displayed graphically as a 'scores' plot. This plot is useful for observing any groupings in the data set. PCA models are constructed using all the samples in the study. Coefficients by which the original variables must be multiplied to obtain the PC are called loadings. The numerical value of a loading of a given variable on a PC shows how much the variable has in common with that component (Massart *et al.*, 1988). Thus for NMR data, loading plots can be used to detect the metabolites responsible for the separation in the data. Generally, this separation takes place in the first three principal components (PC1, PC2 and PC3).

PCA is a visual method that facilitates separation of plant groups according to overall metabolic fingerprints. Analysis of variance (ANOVA) of individual principal components (dependent factors) provides statistical separation of plant groups. For identifying differences in expression of individual compounds, PCA is an exploratory method that indicates which compounds are most responsible for separation of different plant groups. The method therefore has no statistical power to indicate whether compounds are expressed in higher amounts in some groups than others. To test for significant differences in the expression of compounds that are important for plant group differentiation according to PCA, we applied one-way ANOVAs. Quantities of metabolites were expressed in relative intensity (based on integration under spectral peaks, and relative to the internal standard TSP), because absolute concentration is difficult to determine using the analytical techniques applied here. For ANOVAs, plant group (*S. jacobaea*, *S. aquaticus*, hybrids) were defined as fixed factors.

Results

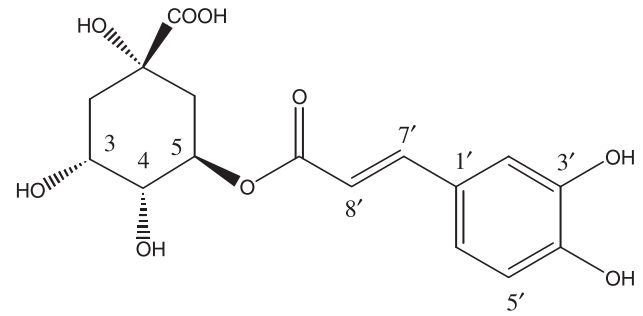
Visual inspection of the NMR spectra (Fig. 1) illustrates that *S. jacobaea* and *S. aquaticus* differ considerably in the overall pattern of metabolites detected. *Senecio aquaticus* exhibits much greater complexity in the carbohydrate region of the spectrum than does *S. jacobaea*, and *S. jacobaea* has stronger signals in the phenolic region of the spectra. Based on initial inspection, hybrids appeared to be intermediate.

Senecio aquaticus, *S. jacobaea* and hybrids were well differentiated based on PCA of quantitative metabolite expression (see Figs 3, 4). A variety of metabolites were responsible for the differentiation, including alanine, chlorogenic acid (Fig. 2), flavonoids (such as quercetin and kaempferol), fumaric acid, glucose, jacaranone analogues (Fig. 2), malic acid, pyrrolizidine alkaloids (Fig. 2), and sucrose. Based on matching of J-resolved spectral patterns, variation among *S. jacobaea* individuals was higher than variation among *S. aquaticus* or hybrid individuals (data not shown).

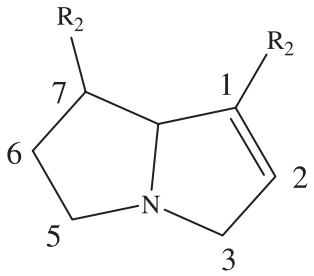
The first three principal components explained 70.3% of the variance. Plant groups could not be differentiated based on PC2. A score plot of PC1 vs PC3 (Fig. 3) shows that species were well separated from one another by both PC1 (ANOVA; $F = 15.299$, $P < 0.000$) and PC3 (ANOVA; $F = 5.402$, $P = 0.013$). Hybrids were not clearly intermediate to parental species based on PCA, as a number of hybrids had PC values that fell outside the range of parental individuals.

The position of peaks on the loading plots (Fig. 4) corresponds to chemical shifts of NMR signals. The intensity of peaks on the loading plots indicates the strength of the correlation between the signals and the principal component. Positive peak values indicate positive correlations, while negative peak values indicate negative correlations. Samples with higher PC values thus accumulate relatively larger amounts of metabolites represented by intense, positive signals on the loading plots, and relatively smaller amounts of metabolites represented by intense, negative signals on the loading plots. For example, *S. aquaticus* and hybrids have significantly higher PC1 values compared with *S. jacobaea* (Tukey's test, data not shown). Examination of the loading plot of PC1 (Fig. 4a) shows that chlorogenic acid, jacaranone, glucose and sucrose are positively correlated with PC1. *Senecio aquaticus* and hybrids thus contain more of these metabolites compared with *S. jacobaea*. Conversely, jacaranone analogues, fumaric acid and several oligosaccharides have negative peak values on the loading plot, and are therefore expressed in lower quantities in *S. aquaticus* or hybrids than in *S. jacobaea*.

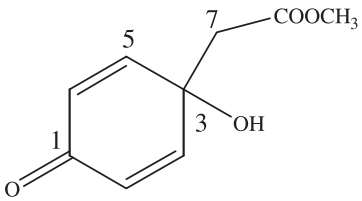
Similarly, hybrids are significantly separated from *S. aquaticus* based on PC3. Hybrids have lower PC3 (Fig. 3) values compared with *S. aquaticus* (Tukey's test, data not shown). The loading plot (Fig. 4b) shows that chlorogenic acid, jacaranone analogues, and several oligosaccharides are negatively correlated with PC3 and thus accumulate more in hybrids, although these differences were shown to be insignificant in later statistical tests (Fig. 5). Sucrose and succinic acid were less expressed in hybrids than in *S. aquaticus*, but these differences were again insignificant in subsequent *post hoc* tests (Fig. 5).



Chlorogenic acid



Pyrrolizidine moiety



Jacaranone

Fig. 2 Chemical structures of chlorogenic acid, unsaturated pyrrolizidine moiety and jacaranone.

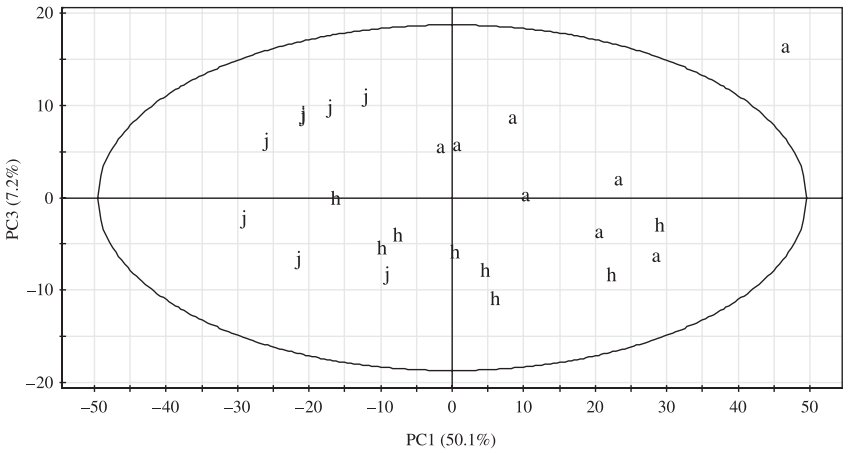


Fig. 3 Score plot of principal component analysis of *Senecio aquaticus* (a), *Senecio jacobaea* (j), and F_1 hybrids (h) between the two species. Ellipse represents Hotelling T2 with 95% confidence in score plots.

Overall, hybrids never expressed higher concentrations of differentiating metabolites than parental species (Fig. 5), even expressing lower amounts of some metabolites, including the amino acids threonine and alanine (Fig. 5b), and phenolics including flavonoids and chlorogenic acid (Fig. 5a). There was no evidence that unique metabolites were expressed in hybrids or either parental species.

Discussion

We found that parental species were well distinguished from each other based on the metabolome, although we did not identify any metabolites that are unique to either parental species in this analysis. Moreover, hybrids can be well distinguished from both parental species based on metabolite expression, and did not cluster intermediately to parents on the basis of PCA.

Hybrids demonstrated differential expression of a number of the metabolites studied here, the trend indicating that metabolite concentration was often reduced in hybrids in relation to parents. Reduction in concentration of some metabolites may result from a higher accumulation of biomass in hybrids vs parents (Kirk *et al.*, 2005), rather than reduced production *per se*. Reduction in concentration of some metabolites might occur if the ratio of metabolite-producing organs to total plant biomass decreases (Hol *et al.*, 2003), but it is not

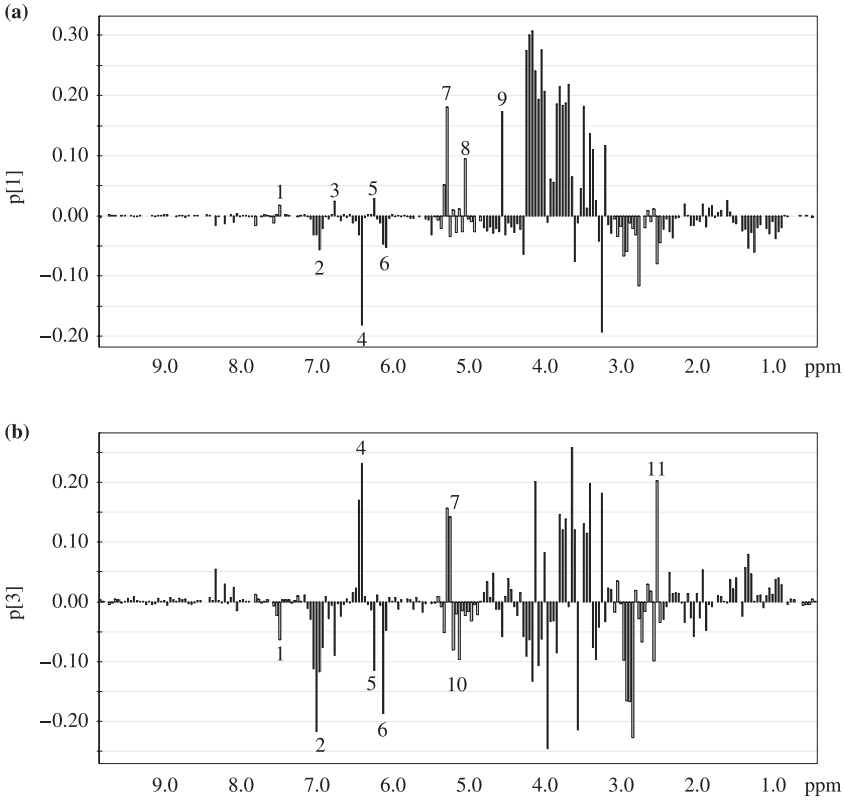


Fig. 4 Loading plots of principal component analysis of *Senecio* species: (a) PC1; (b) PC3. 1, H-8′ of chlorogenic acid; 2, H-2 and H-6 of jacaranone analogues; 3, H-5′ of chlorogenic acid; 4, H-β of fumaric acid; 5, H-7′ of chlorogenic acid; 6, H-6 of H-3 and H-5 of jacaranone analogues; 7, H-1 of sucrose; 8, H-1 of α-glucose; 9, H-1 of β-glucose; 10, H-1 of oligosaccharides; 11, H-β of succinic acid. Pyrrolizidine alkaloids and flavonoids are not shown.

possible to draw conclusions about whether this occurs without specific knowledge about the production and accumulation of individual compounds.

NMR proved to be a good analytical technique to detect a wide range of primary and secondary metabolites. It is generally accepted that a single analytical technique will not provide sufficient visualization of the metabolome, and therefore multiple technologies are needed for a comprehensive view (Summer *et al.*, 2003). However, sometimes limits to the amount of biological material available, time or funding limits, or metabolic instability force us to choose an optimum analytical tool for metabolomic profiling. Therefore it is preferable to use a wide-spectrum chemical analysis technique which is rapid, reproducible and stable over time, while requiring only very basic sample preparation. Nuclear magnetic resonance spectroscopy is a technique that meets those requirements.

One of the major drawbacks of NMR is that it fails to detect compounds present in relatively low concentrations. We did not identify any unique metabolites in any of the plant groups studied here. This result was probably an artefact of the NMR analysis. First, NMR necessarily detects only the most abundant metabolites in a sample. However, abundant metabolites may have a high fitness value to plants, and may be much less likely to exhibit qualitative variation in expression between genotypes and species. Second, NMR may detect general groups of secondary metabolites, but not small structural differences within such groups. For example, in this study we detected the basic structure (moiety) of unsaturated PAs, which are known to be important for plant–insect interactions in *Senecio*. Yet it is known that *S. jacobaea* alone can contain up to 10 different PA structures. Moreover, in other studies we found that *S. aquaticus* produces at least one PA that is not produced by *S. jacobaea* (Kirk *et al.*, 2004; unpublished data), and that F_1 hybrids produce one PA that is unique to both parental species (unpublished data). In general, novel compounds produced by hybrids are likely to be present in small concentrations, and may represent structural variations within metabolite classes that cannot be detected by broad-scale metabolomic approaches using NMR. This is the major shortcoming of using metabolomic profiling techniques to study metabolite expression in hybrids vs parental species.

NMR-based metabolomic profiling, as described here, may be best applied when there is little previous knowledge of the metabolites expressed by a group of study organisms, or when researchers are seeking to identify quantitative differences in major groups of primary and secondary metabolites. This study, for instance, provides a basis for more detailed study of a number of secondary metabolite classes, including flavonoids and jacaranone derivatives, which have never been reported from the *Senecio* species studied here. Broad-spectrum NMR profiling is a powerful approach for researchers interested in major quantitative differences between plant groups (Ward *et al.*, 2003), or between plant subjected to differing ecological conditions (Choi *et al.*, 2004).

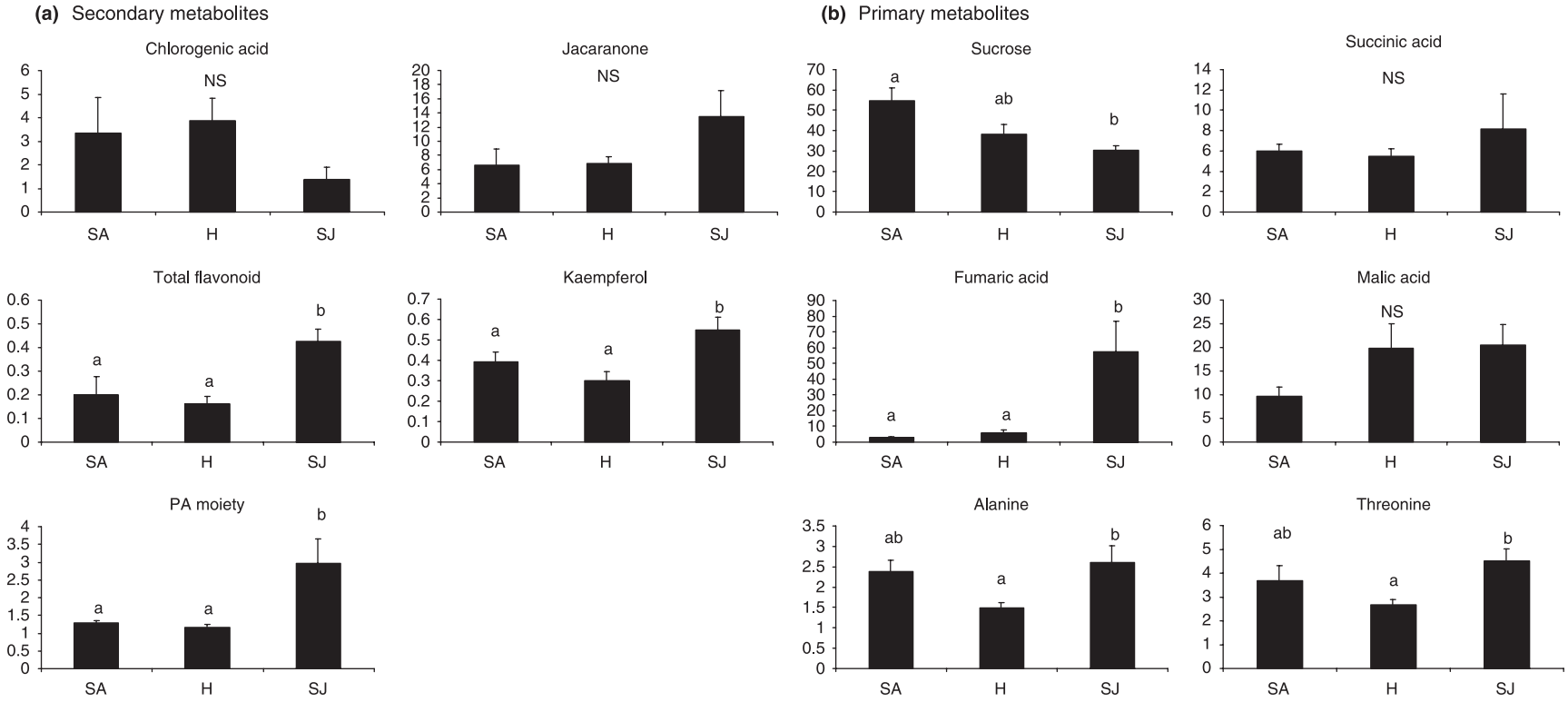


Fig. 5 Quantitative expression of secondary (a) and primary (b) metabolites in *Senecio jacobaea* (SJ), *Senecio aquaticus* (SA), and F_1 hybrids (H). Total flavonoids are shown, as well as kaempferol, which was putatively identified as one of the flavonoid components. Letters indicate significant differences between groups (Tukey's test, $P < 0.05$; NS, no significant difference). The *y*-axis represents peak intensity relative to the internal standard TSP. Relative concentrations can be compared between plant individuals or groups, but not between different compounds. Bars represent SE.

It should also be noted that a number of secondary metabolites that may be involved in plant–herbivore interactions were detected in this study. Pyrrolizidine alkaloids have been well studied in *Senecio* species, are known to play a role in plant–herbivore interactions (Macel, 2003), and can reduce the growth of plant pathogens (Hol & van Veen, 2002). Jacaranone analogues (benzoquinoids), identified for the first time in the species studied here, have been isolated from several other *Senecio* species (Lajide *et al.*, 1996; Torres *et al.*, 2000; Xu *et al.*, 2003). Jacaranone analogues have been shown to have insecticidal activity against adult house flies (Xu *et al.*, 2003), and can act as a growth inhibitor for the generalist herbivore *Spodoptera litura* (Lajide *et al.*, 1996). Furthermore, chlorogenic acid has, to our knowledge, never been isolated from *Senecio* species, although it is a ubiquitous compound in plants. Chlorogenic acid may have a mixed effect on herbivores: Bi *et al.* (1997) found no effect of chlorogenic acid on a generalist and a specialist caterpillar feeding on tobacco; while Felton *et al.* (1992) found that the same compound reduced the nutritional quality of proteins for the generalist herbivore *Spodoptera exigua* (Felton *et al.*, 1992). Additionally, phenolic compounds such as chlorogenic acid may play a role in resistance to sucking insects (Miles & Oertli, 1993). Finally, flavonoids are known to convey herbivore resistance by inhibiting growth of Lepidopteran larvae (Mallikarjuna *et al.*, 2004), and can be sequestered by specialist herbivores (Wiesen *et al.*, 1994), indicating that these compounds can play a role in plant interactions with both specialist and generalist herbivores. Other major structural groups found in other *Senecio* species, including sesquiterpene lactones and polyacetylenes, were not apparent in the NMR spectra, and thus do not constitute a significant component of the metabolome of these species.

The presence of multiple putative defence compounds in the *Senecio* species studied here, as well as differing patterns of resistance of *Senecio* genotypes and species to different herbivores (unpublished data), confirm that these species may have a mosaic of defences that act differentially on different herbivores. It would be interesting to continue detailed studies to determine whether structural variants of flavonoids, jacaranone and PAs occur within parental species and hybrids, and whether hybrids possess unique variants of these compounds. Furthermore, it would be interesting to combine such studies with herbivore resistance tests, to elucidate the role of these secondary metabolites in plant resistance. Overall, *Senecio* is a potentially useful genus for the study of selection on plant defences by multiple herbivores, and this study provides a basis for such future research.

Acknowledgements

Heather Kirk thanks the Natural Resources and Engineering Council of Canada (NSERC) for financially supporting her research. Thanks to Peter Klinkhamer and Klaas Vrieling, who contributed useful suggestions for the manuscript and the science presented therein, and to two anonymous reviewers for useful comments on the manuscript.

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Appendix 1: Technical description of NMR measurements

^{1}H NMR and J-resolved spectra were recorded at 25°C on a 400 MHz Bruker AV-400 spectrometer operating at a proton NMR frequency of 400.13 MHz. Methanol-d_4 was used as the internal lock. Each spectrum consisted of 128 scans requiring 10 min acquisition time with the following parameters: 0.25 Hz per point; pulse width = 90° (6.6 μs); relaxation delay = 5.0 s. A presaturation sequence was used to suppress the residual water signal with low-power selective irradiation at the water frequency during the recycle delay. Free induction decays were Fourier transformed with line broadening LB = 0.3 Hz and spectra were zero-filled to 32 K points. Window functions were optimized for the analysis. The resulting spectra were manually phased and baseline corrected, and calibrated to the internal standard TSP at 0.0 ppm, all using XWIN NMR (ver. 3.5, Bruker). Two dimensional J-resolved ^{1}H-NMR spectra were acquired using eight scans per 32 increments, collected into 16 K data points, using spectral widths of 5.208 kHz in F_2 (chemical shift axis) and 50 Hz in F_1 (spin–spin coupling constant axis). A 1.0 s relaxation delay was employed, giving a total acquisition time of 14.52 min. Data sets were zero-filled to 512 points in F_1 and both dimensions were multiplied by sine-bell functions before double complex Fourier transformation (FT). J-resolved spectra were tilted by 45°, symmetrized about F_1, and then calibrated, all using XWIN NMR. Data were exported as the one-dimensional projection (F_2 axis) of the two-dimensional J-resolved spectra.

Appendix 2: Visual inspection of NMR spectra and assignment of metabolites

^{1}H-^{1}H-COSY (correlated spectroscopy), TOCSY (total COSY), HSQC (heteronuclear single quantum correlation) and HMBC (heteronuclear multiple bond correlation) were measured at 600 MHz Bruker DMX-600 spectrometer operating at a proton NMR frequency of 600.13 MHz. The COSY spectra were acquired with 1.0 s relaxation delay, 6361 Hz spectral width in both dimensions. The HSQC spectra were obtained with 1.0 s relaxation delay, 6361 Hz spectral width in F_2 and 27 164 Hz in F_1. The HMBC spectra were recorded with the same parameters as the HSQC spectrum, except for 30 183 Hz spectral width in F_2. The TOCSY spectra were acquired with 1.0 s relaxation delay, 6361 Hz spectral width in both dimensions.

The major differences between the *Senecio* species are observed in the aromatic region of the ^{1}H-NMR spectra (Figs 3, 5). The cluster of signals distinguished at δ 7.1 and 6.1 was found to be a doublet (J = 9.6 Hz) in ^{1}H-^{1}H-2D-J-resolved spectra, and assigned as H-2, H-3, H-5 and H-6 of jacaranone analogues. HMQC spectra showed that H-2It was confirmed by ^{1}H-^{1}H-COSY and HMBC spectra in which H-2, H-6 at δ 7.1 and H-3, H-5 at δ 6.1 correlated with C-1 at

δ 189.2 and C-4 at δ 69.4. Other phenolic metabolites, such as chlorogenic acids and flavonoids, were detected. Two characteristic *trans* olefinic protons of chlorogenic acids were shown at δ 7.64 (H-8′, *d*, *J* = 15.9 Hz) and δ 6.39 (H-7′, *d*, *J* = 15.9 Hz) (Choi *et al.*, 2004). As a minor signal, H-6 and H-8 of flavonoids were observed at δ 6.32 (*d*, *J* = 2.0 Hz) and δ 6.50 (*d*, *J* = 2.0 Hz). A signal at δ 7.94 was putatively identified to belong to kaempferol, which has a 4′ hydroxyl group. The singlet at δ 6.52 was assigned to be an olefinic proton of fumaric acid, which was confirmed by HMBC in which the proton correlated with the carbonyl signal at δ 176.2.

There was also significant difference in the anomeric signals of carbohydrates such as δ 5.42 (*d*, *J* = 3.8 Hz); δ 5.18 (*d*, *J* = 3.7 Hz); and δ 4.57 (*d*, *J* = 9.5 Hz). These were assigned to be the anomeric protons of sucrose, α-glucose, and β-glucose, respectively (Agrawal, 1992). Another anomeric signal obtained from the fructose moiety of sucrose is also easily distinguishable at δ 4.22 (*d*, *J* = 8.8 Hz). The residual proton signals of the sugars shown in the crowded region (δ 3.0–4.0) were assigned by comparison of ^{1}H-NMR spectra of the reference compounds ^{1}H-^{1}H-COSY and TOCSY spectra. Other anomeric signals detected at δ 5.35–5.45 were assigned as anomeric signals of oligosaccharides, which was confirmed by HMBC and TOCSY spectra. In the region δ 5.40–5.90, H-2 of unsaturated pyrrolizidine alkaloids was observed as minor signals.

The complexity in the range of δ 2.5–3.0 was also clearly resolved by two-dimensional J-resolved spectra. The splitting pattern of this region was deconvoluted by the J-resolved spectrum, indicating signals belonging to malic acid at δ 2.80 (*dd*, *J* = 16.6 Hz, 4.7 Hz) and δ 2.61 (*dd*, *J* = 16.6 Hz, 6.6 Hz), and succinic acid at δ 2.54 (*s*).