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Research Article

Application of methyl jasmonate and salicylic acid lead to contrasting effects on the plant's metabolome and herbivory

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ABSTRACT

Phytohormone applications are used to mimic herbivory and can induce plant defences. This study investigated (i) metabolomic changes in leaf tissues of *Jacobaea vulgaris* and *J. aquatica* after methyl jasmonate (MeJA) and salicylic acid (SA) applications and (ii) the effects on a leaf-chewing, a leaf-mining and a piercing-sucking herbivore. MeJA treated leaves showed clearly different metabolomic profiles than control leaves, while the differences in metabolomic profiles between SA treated leaves and control leaves were less clear. More NMR peaks increased than decreased after MeJA treatment while this pattern was reversed after SA treatment. The leaf-chewing (*Mamestra brassicae*) and the leaf-mining herbivores (*Liriomyza trifolii*) fed less on MeJA-treated leaves compared to control and SA-treated leaves while they fed equally on the latter two. In *J. aquatica* but not in *J. vulgaris*, SA treatment reduced feeding damage by the piercing-sucking herbivore (*Frankliniella occidentalis*). Based on the herbivory and metabolomic data after phytohormone application, we made speculations as follows: For all three herbivore species, plants with high levels of threonine and citric acid showed less herbivory while plants with high levels of glucose showed more herbivory. Herbivory by thrips was lower on plants with high levels of alanine while it was higher on plants with high levels of 3,5-dicaffeoylquinic acid. The plant compounds that related to feeding of piercing-sucking herbivore were further verified with previous independent experiments.

1. Introduction

Plants have evolved different strategies, including chemical and mechanical defences, to cope with attacks from herbivores and pathogens [1,2]. Chemical defences are mainly based on secondary metabolites (SMs) derived from different chemical origins, often characteristic for certain plant taxa and effective at least against generalist herbivores [3]. Mostly shifts in chemical composition upon attack are herbivore-specific and depend to some degree on the feeding mode of the herbivore [4,5].

Induction of chemical defense after attack is mediated by phytohormones such as jasmonate (JA), ethylene (ET) and salicylic acid (SA). As a general rule, the JA pathway, which frequently acts synergistically

with ET, is up-regulated if the plant is attacked by chewing-biting herbivores, cell-content feeders and necrotrophic pathogens [6,7], while the SA pathway is activated in response to piercing-sucking insects and biotrophic pathogens [7,8]. Therefore, it is predicted that the compounds activated by the JA pathway help the plant to resist chewing-biting herbivores and cell-content feeders while the compounds activated by the SA pathway help the plant to resist piercing-sucking herbivores. JA and SA pathways are not distinct from each other but interact by a complex network of regulatory interactions [9,10], including priming [11,12], complementary additive or synergistic effects [13,14] and mutual antagonism [15,16].

Direct exogenous application of phytohormones has been commonly used to simulate attack by herbivores and to analyse the plant responses

Abbreviations: NMR, nuclear magnetic resonance; MeJA, methyl jasmonate; JA, jasmonic acid/jasmonate; SA, salicylic acid; CON, control; PCA, principle component analysis; PA, pyrrolizidine alkaloid; SM, secondary metabolites; MS, Murashige and Skoog; 3,5-DCQA, 3,5-dicaffeoylquinic acid; Ile, isoleucine; VOC, volatile organic compound.

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to herbivory [17]. In *Brassicae* and *Nicotiana* species, glucosinolate and nicotine concentrations increased in response to JA application [18,19]. SA application induced resistance against rice stink bugs [20]. The artificial application of JA reduced thrips-associated damage in a whole plant no-choice bioassay of tomato [21]. Most of these studies showed the effects of JA or SA application on the production of SMs or the performance of herbivores. Only few studies aimed at coupling the changes in metabolic profiles upon hormone application with the changes in herbivore feeding [22,23]. In addition, most studies were targeted at one specific group of plant SMs while other chemical compounds, which might also be important to defense, were neglected [24, 25].

In this study, we investigated how metabolic profiles were affected by exogenous application of methyl jasmonate (MeJA) and SA. We also examined how these induced metabolic profiles were associated with feeding by herbivores with different feeding modes. We addressed these questions with two plant species known for their pyrrolizidine alkaloids (PAs), common ragwort, *Jacobaea vulgaris* (syn. *Senecio jacobaea*) and marsh ragwort, *Jacobaea aquatica* (both Asteraceae). Previously, we showed that, while the total PA concentration in these plant species was not affected by root application of MeJA, the composition of PAs shifted significantly from senecionine- to erucifoline-like PAs [26]. However, it remained unclear to what extent other plant chemical components are affected by phytohormone application. A leaf-chewing herbivore, cabbage moth, *Mamestra brassicae* (Lepidoptera: Noctuidae), a leaf-mining herbivore, celery leafminer, *Liriomyza trifolii* (Diptera: Agromyzidae) and a piercing-sucking herbivore, western flower thrips, *Frankliniella occidentalis* (Thysanoptera: Thripidae) were used in this study. Our experiments were designed to answer the following questions: (1) How do the plant metabolic profiles change after MeJA and SA application to the leaves? (2) Is the feeding damage of the leaf-chewing herbivore and the leaf-mining herbivore reduced by MeJA application? (3) Is the feeding damage of the piercing-sucking herbivore reduced by SA application? (4) Are the changes in the plant metabolites related to the changes in herbivore feeding? (5) If so, which compounds are associated with reduced or increased feeding levels of these three herbivores? The compounds that potentially affected the feeding of *F. occidentalis* were further investigated by combining the results of the present experiment with those of two previous experiments [27,28].

2. Materials and methods

2.1. Plants and insects

Jacobaea vulgaris and *Jacobaea aquatica* individuals used in this study are the parental genotypes of a well-studied hybrid cross [29]. *J. vulgaris* was derived from seeds collected at the Meijendel Nature Reserve (52°7'54"N, 4°19'46"E, The Netherlands), and *J. aquatica* was derived from seeds collected at the Zwanenwater Nature Reserve (52°48'38"N, 4°41'7"E, The Netherlands). *J. vulgaris* is attacked by over 60 species of herbivores and grows in dry and sandy soils while *J. aquatica* is fed on by relatively few herbivores and grows in marshy environments [30]. Genotypes of both species are maintained in tissue culture and can easily be cloned to produce replicates. Each plantlet was grown in 5 mL of Murashige and Skoog (MS-0) medium to induce a fully developed root system in a climate room (humidity 50 %, light 16 h /dark 8 h, 20 °C/20 °C).

Eggs of the chewing herbivore *Mamestra brassicae* were obtained from the Entomology Lab, WUR (Wageningen, The Netherlands) and caterpillars were reared on cabbage in a climate room (humidity 50 %, light 16 h /dark 8 h, 20 °C/20 °C). The leafminer *Liriomyza trifolii*, with a cell-content feeding mode, and the thrips, *Frankliniella occidentalis*, with a piercing-sucking feeding mode, were both from cultures maintained in our lab. Both species were reared on chrysanthemum (*Dendranthema grandiflora*) in a climate room (humidity 50 %, light 16 h /dark 8 h, 20 °C/20 °C).

2.2. Phytohormone treatments

After two weeks of growth in MS-0 medium, the shoots were randomly assigned to be dipped for 30 s in one of the following treatments: (1) Control (CON), 0.1 % Triton water solution; (2) 5 mM Methyl jasmonate (MeJA) dissolved in 0.1 % Triton solution; (3) 5 mM Salicylic acid (SA) dissolved in 0.1 % Triton solution. Ten individuals per plant species were subjected to each treatment. After dipping, the plants were placed on a paper tissue to absorb solution left on the leaves, and put in new tubes with MS-0 medium. After two weeks, the second leaf from the bottom of the plant was removed, three leaf discs (1.2 cm diameter) were obtained from this leaf and then used in one of the bioassays described below (one leaf disc per plant was used for each herbivore assay). The remaining leaf material was wrapped in aluminium foil, placed in liquid nitrogen and then kept in a -80 °C freezer until freeze drying.

2.3. Bioassays with herbivores

Non-choice feeding bioassays to test herbivore feeding performances were conducted in Petri dishes (60 mm * 15 mm) lined with a layer of agar to maintain a constant humidity. In each petri dish, a leaf-disc was gently pressed on the agar with the abaxial side up. One hundred and eighty petri dishes were prepared for the three herbivore bioassays: two plant species (*J. vulgaris* and *J. aquatica*) * three treatments (CON, MeJA and SA) * three herbivore bioassays * ten replicates = 180. These herbivore bioassays were conducted in a climate room (humidity 50 %, light 16 h /dark 8 h, 20 °C/20 °C).

In the bioassay with *M. brassicae*, the 2nd instar caterpillars with similar body size were selected. One caterpillar was released in each Petri dish. The caterpillars were allowed to feed for four hours. The damaged leaf-discs were measured with image analysis software of WinFolia Pro (Regent Instruments Inc., Québec City, Canada). The area of the leaf surface eaten was calculated by subtracting the remaining leaf area from that of an intact leaf disc.

For the bioassay with *L. trifolii*, two female leafminers were released in one Petri dish. After 24 h, the number of feeding punctures were counted under a stereo microscope.

In the thrips bioassay with *F. occidentalis*, four female thrips were released in each Petri dish. The thrips were allowed to feed for seven days. The amount of silver damage for each leaf was visually scored in mm² according to the methods developed by Leiss et al. [31].

2.4. Metabolomic analysis

Freeze-dried plant material was extracted and analysed by NMR spectroscopy according to Kim et al. [32]. Briefly, 20 mg of lyophilized sample was placed into a 2.0 mL microtube and 0.5 mL methanol-d₄ and 0.5 mL D₂O (KH₂PO₄ buffer, pH = 6.0) containing 0.005 % TMSP-d₄ (trimethyl silyl propionic acid sodium salt-d₄, w/v, Sigma-Aldrich, Zwijndrecht, the Netherlands) were added. The mixture was extracted at room temperature for 20 min with ultrasonication (Branson 5510E-MT, Branson Ultrasonics, Danbury, CT, USA) and subjected to centrifugation at 17,000 ×g at room temperature for 5 min. 300 µl of the supernatant was transferred to a 3 mm NMR tube and analysed.

The ¹H-NMR spectra were recorded using a Bruker DMX 600 spectrometer (Bruker, Karlsruhe, Germany). For each sample, 64 scans were recorded with the following parameters: 0.167 Hz/point, pulse width (PW) = 4.0 µs, acquisition time (AQ) = 3.17 s, dummy scans (DS) = 4 and relaxation delay (RD) = 1.5 s. Free induction decays (FIDs) were Fourier transformed with line broadening (LB) = 0.3 Hz. Phase adjustment and baseline correction were applied manually.

The AMIX software (Bruker) was used to convert the ¹H-NMR spectra to an ASCII file. The spectra, which were measured in chemical shift (ppm), were binned (bucketed) into bins of equal width (0.04 ppm). Spectral intensity was scaled to internal standard (TMSP) and reduced to

integrated regions of equal width (0.04 ppm) corresponding to the region of δ 10.00–0.40. The region of δ 4.88–4.72 was excluded from the analysis because of the residual signal of H₂O and CH₃OH-d₄, respectively. The regions of δ 10.0–7.68 and δ 0.8–0.4 were excluded from the analysis because these contained no significant peaks. The metabolites were identified based on a library with one-dimensional and two-dimensional NMR spectra of more than 300 compounds, which is available in our laboratory [32].

2.5. Statistical analysis

Principal component analysis (PCA) of the NMR spectra was performed with the SIMCA-P (ver. 13.0, Umetrics, Umeå, Sweden) to determine if treatments and plant species differed from each other. Differences in herbivore feeding among control, MeJA treatment and SA treatment were evaluated using Kruskal-Wallis analysis, followed by Dunn's post hoc tests. The peak intensity of NMR bins representing identified compounds were compared between each phytohormone treatment and the control group with Mann-Whitney U-tests for each species. *P*-values of the bins in a specific pairwise comparison was adjusted according to Benjamini & Hochberg method [33] to correct for multiple testing. All the analyses were performed in R 3.6.0.

2.6. Correlation with datasets of previous studies in thrips bioassays

To identify the compounds involved in thrips resistance, we combined and analysed the results of two of our previous experiments. The combination of the two independent experiments reduces the chance of false positives. In brief, a segregating population of 94 F₂ hybrids were obtained from a cross between *J. vulgaris* and *J. aquatica*. After culturing for six weeks, the shoots were cut for NMR measurements [28] or used for a feeding bioassay with thrips [27] (Fig. S1). The relationships between the amount of silver damage by thrips and NMR signals for the 94 F₂ lines were analysed with Spearman rank correlations. *P*-values were adjusted according to Benjamini & Hochberg method [33] for multiple testing. NMR bins that were significantly correlated with thrips damage in the F₂ studies were then compared with the significant NMR bins of the SA application in *J. aquatica*. We selected the NMR bins that were increased after SA application and negatively correlated with silver damage in the F₂ hybrids (potential resistance-conferring compounds), and the NMR bins that were reduced after SA application and positively correlated with silver damage in the F₂ hybrids (potential susceptibility-conferring compounds). Compounds represented by these selected bins were identified as described in Section 2.4.

3. Results

3.1. Effects of phytohormone treatments on the NMR metabolite profile of leaf tissue

A principle component analysis (PCA) of the ¹H-NMR peaks from

Table 1

Summary of up- or down-regulated NMR bins with a changed peak intensity compared to the control after methyl jasmonate (MeJA) and salicylic acid (SA) treatments in *Jacobaea vulgaris* and *Jacobaea aquatica*.

Plant species	<i>J. vulgaris</i>		<i>J. aquatica</i>	
	MeJA	SA	MeJA	SA
Treatments	MeJA	SA	MeJA	SA
Total bins	170	170	170	170
Down-regulated bins	65	117	42	116
Up-regulated bins	105	53	128	54
Significant bins ^a	76	7	122	76
Significant down-regulated bins	19	6	25	54
Significant up-regulated bins	57	1	97	22

^a The number of NMR bins that are significantly different from the control after adjusting for multiple comparisons by the Benjamini & Hochberg method.

J. vulgaris showed that, 63.1 % of the variation was explained by the first two axes (Fig. 1a) while the third axis explained 9.2 %. In *J. aquatica*, 64.7 % of the variances was explained by the first two axes (Fig. 1b) and another 10.0 % was explained by the third axis. PCA plots of the first two components showed that the plants treated with MeJA were separated from the control and SA treated plants for both plant species. Although the control plants were well separated from the SA treated plants in *J. aquatica* (Fig. 1b), they showed some overlap in *J. vulgaris* (Fig. 1a).

Compared to the control group, the NMR signal in more bins were significantly up-regulated after the MeJA treatment while the NMR signal in more bins were significantly down-regulated after the SA treatments in both plant species (Tables 1, S1). MeJA treatment resulted in 3.0 and 3.7 times more significantly up-regulated than down-regulated bins in *J. vulgaris* and in *J. aquatica* respectively (Table 1). SA application led to 6.0 and 2.5 times more down-regulated than up-regulated bins in *J. vulgaris* and *J. aquatica* respectively (Table 1).

MeJA treatment caused significant changes in NMR signal in 76 bins and 122 bins and SA treatment caused significant changes in NMR signal in 7 bins and 76 bins in *J. vulgaris* and *J. aquatica*, respectively. This means that, for the MeJA and SA treatments, 1.6 and 10.9 times more significantly bins with a changed NMR signal were detected in *J. aquatica* than that in *J. vulgaris* respectively (Tables 1, S1).

3.2. Effects of phytohormone treatments on herbivore feeding

The leaf area eaten by chewing herbivore *M. brassicae* significantly differed between phytohormone treatments for both plant species (Kruskal Wallis test, *J. vulgaris*: $\chi^2 = 14.551$, *df* = 2, *P* < 0.001;

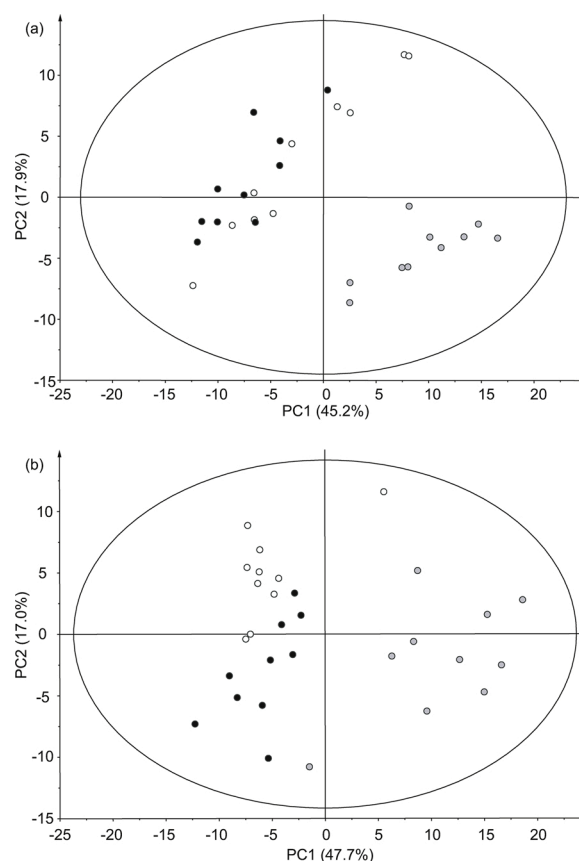


Fig. 1. Plot of principal components 1 and 2 based on PCA of the signal intensity of 170 ¹H-NMR bins of *Jacobaea vulgaris* (a) and *Jacobaea aquatica* (b). White dots represent control plants, grey dots represent plants treated with methyl jasmonate (MeJA) and black dots represent plants treated with salicylic acid (SA), *n* = 10 for each treatment.

J. aquatica: $\chi^2 = 14.837$, $df = 2$, $P < 0.001$). The consumed leaf area was significantly lower on plants treated with MeJA than on plants treated with SA and the control plants (Fig. 2a). The leaf area consumed by *M. brassicae* in the SA treatment did not differ from the control treatment.

Similarly to *M. brassicae*, the number of leaf punctures by the leaf-mining herbivore *L. trifolii* differed significantly between the phytohormone treatments for both plant species (Kruskal Wallis test, *J. vulgaris*: $\chi^2 = 8.240$, $df = 2$, $P = 0.016$; *J. aquatica*: $\chi^2 = 7.747$, $df = 2$, $P = 0.021$). The number of leaf punctures was significantly reduced in the MeJA treatment plants compared to the SA treated plants and the control plants (Fig. 2b); the number of leaf punches were similar in SA treated and control plants.

The amount of silver damage by the piercing-sucking thrips *F. occidentalis* was significantly affected by hormonal treatment in *J. aquatica* (Kruskal Wallis test, $\chi^2 = 6.938$, $df = 2$, $P = 0.031$) but not in *J. vulgaris* (Kruskal Wallis test, $\chi^2 = 0.089$, $df = 2$, $P = 0.957$). In *J. aquatica* the silver damage was significantly reduced on the plants treated with SA. MeJA treated plants did not differ significantly from the control although damage was reduced 50 % compared to the control (Fig. 2c).

3.3. Variation of identified compounds after phytohormone treatments

In total, 16 compounds were identified from the NMR spectra in the leaf tissue (Table 2), including 13 primary metabolites (PMs) and three secondary metabolites (SMs). The 13 PMs included two sugars (sucrose

Table 2

^1H chemical shift (d) of metabolites present in leaves of *Jacobaea vulgaris* and *Jacobaea aquatica* identified by 1D NMR spectra in MeOH-d_4 .

No	Compounds	Chemical shift (ppm)
1	Alanine	δ 1.48 (d)
2	Asparagine	δ 2.94 (d), 2.96 (d)
3	Aspartate	δ 2.71 (d), 2.68 (d)
4	Chlorogenic acid	δ 7.61 (d)
5	Choline	δ 3.22 (s)
6	Citric acid	δ 2.55 (s)
7	3,5-dicaffeoylquinic acid	δ 6.90 (d), 6.88 (d), 6.49 (d)
8	Fumaric acid	δ 6.53 (s)
9	Glucose	δ 5.18 (d)- α , δ 4.59 (d)- β
10	Glutamate	δ 2.40 (m)
11	Glutamine	δ 2.46 (m), 2.13 (m)
12	Isoleucine	δ 0.96(t)
13	Jacaranone	δ 6.24 (d), 7.14 (d)
14	Sucrose	δ 5.41 (d)
15	Threonine	δ 1.34 (d)
16	Valine	δ 1.06(d), 1.00 (d)

and glucose), eight amino acids (asparagine, aspartate, glutamine, glutamate, alanine, threonine, valine and isoleucine), fumaric acid, citric acid and choline. The three SMs consisted of jacaranone, chlorogenic acid and 3, 5-dicaffeoylquinic acid (3,5-DCQA).

Threonine, valine and isoleucine in *J. vulgaris* and *J. aquatica* increased after either MeJA or SA treatment, although the increase was not statistically supported for valine in the SA treatment of *J. aquatica*, the MeJA treatment of *J. vulgaris* and for all three amino acids in the SA

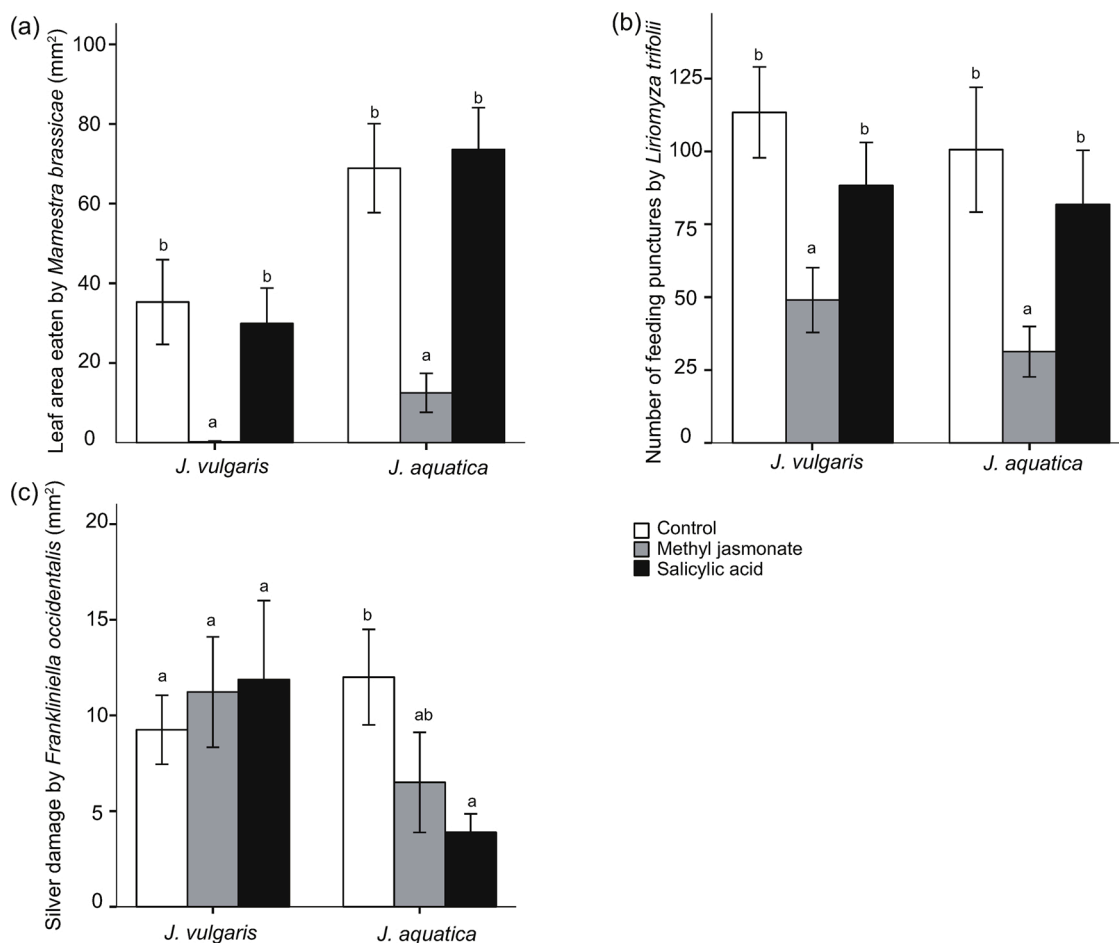


Fig. 2. Feeding damage (\pm SE) by three different herbivores on leaf punches 2 weeks after treatment with MeJA and SA in *Jacobaea vulgaris* and *Jacobaea aquatica*. (a) caterpillar *Mamestra brassicae*, (b) leaf miner *Liriomyza trifolii*, (c) thrips *Frankliniella occidentalis*. Different letters indicate significant differences at $P < 0.05$ between treatment groups with Kruskal Wallis test within a species followed by a Dunn post hoc test, $n=10$ per treatment.

treatment of *J. vulgaris* (Fig. 3, Table S1). MeJA application induced significant increases in jacaranone, asparagine and citric acid, and SA application induced a significant increase in alanine in both plant species. In addition, 3,5-DCQA and citric acid in *J. aquatica* increased significantly after MeJA and SA application respectively (Fig. 3, Table S1).

Chlorogenic acid decreased after treatment with MeJA and SA in both plant species (Fig. 3). Glucose and glutamate significantly decreased after MeJA treatment in both plant species. Jacaranone, 3,5-DCQA, sucrose and fumaric acid decreased significantly after SA application in *J. aquatica*, and they also decreased in *J. vulgaris* but not significantly (Fig. 3).

3.4. Candidate compounds potentially involved in herbivore resistance

3.4.1. Candidate compounds potentially affecting leaf-chewing *Mamestra brassicae* and leaf-mining *Liriomyza trifolii*

In both *J. vulgaris* and *J. aquatica*, feeding by *M. brassicae* and *L. trifolii* significantly decreased in MeJA treated plants. If the peak intensity of identified compounds increased after MeJA application, they were potentially associated with lower feeding of *M. brassicae* and *L. trifolii*; if the peak intensity of identified compounds decreased after MeJA application, they were potentially associated with an increased herbivory by the two herbivores. Therefore, higher concentrations of jacaranone, asparagine, threonine, isoleucine and citric acid were potentially associated with lower feeding (Fig. 4a–j), while higher concentrations of glucose and glutamate were associated with higher feeding (Fig. 4k–n).

3.4.2. Candidate compounds potentially affecting piercing-sucking *Frankliniella occidentalis*

Feeding by *F. occidentalis* significantly decreased in SA treated leaves in *J. aquatica* only, and therefore we focus only on this plant species hereafter. Following the same reasoning as above, increased concentrations of alanine, threonine, isoleucine and citric acid were associated with less herbivory while increased levels of jacaranone, 3,5-DCQA, sucrose, glucose and fumaric acid were associated with more herbivory.

For *F. occidentalis* we combined the results of the present study with the results of two previous studies. In the latter, thrips damage of 94 F2 lines from a cross using the same parental genotypes as in the present study was correlated with their metabolomic NMR patterns (For a description of that cross see [27,28]). In this correlative study, the signals of 17 bins were positively correlated and the signals of 25 bins were negatively correlated with thrips silver damage (Tables 3, S2). Six of these bins also showed a significant increase in the NMR signals after SA application in the current experiment and for three of these the NMR signals were identified as alanine, threonine and citric acid. Eight of 17 positive correlated bins showed a significant decrease in NMR signals after SA application in the current experiment and of these, the NMR signals were identified as 3,5-DCQA and three bins were broadly identified as “sugars”. We were unable to further identify these to specific sugars. The NMR signal of three up-regulated bins and three down-regulated ones were low and could not be identified (Table S2). Therefore, based on these combined results of the present experiment and the previous correlative study, alanine, threonine and citric acid were identified as candidate compounds involved in plant resistance to

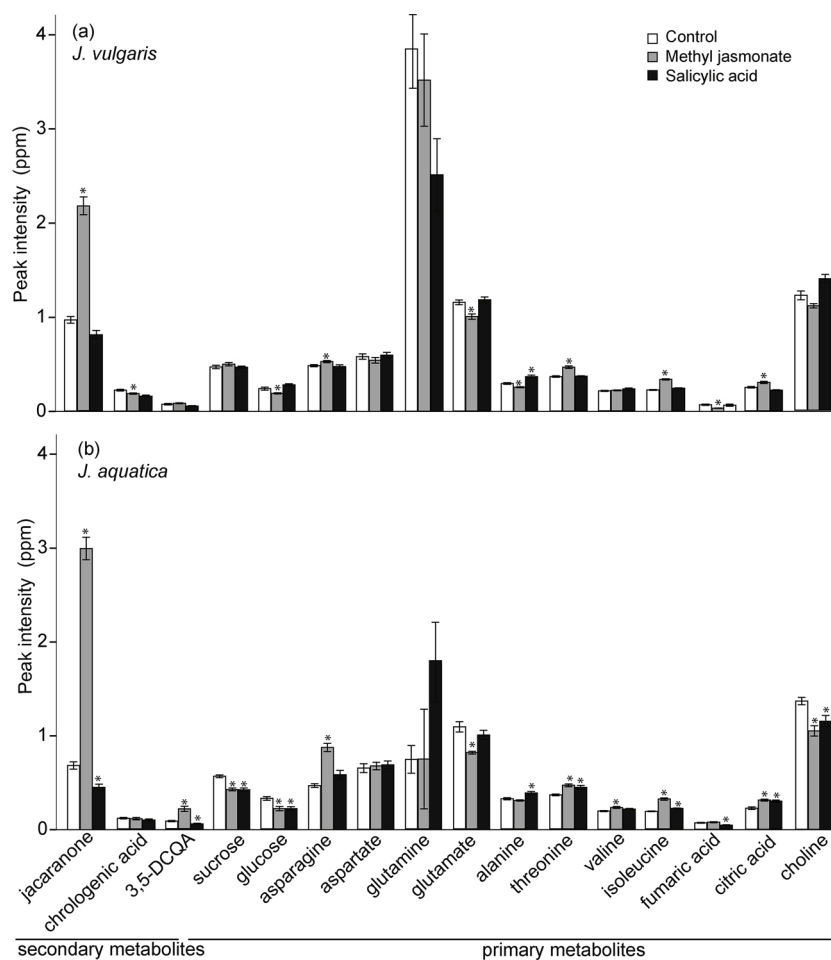


Fig. 3. Semi-quantitative expression of secondary and primary metabolites identified in *Jacobaea vulgaris* (a) and *Jacobaea aquatica* (b) leaves after phytohormone application. The Y-axis represents peak intensity relative to the internal standard TMSP. Asterisks indicate metabolites that differed significantly from the control after correction for multiple testing with the Benjamini-Hochberg method.

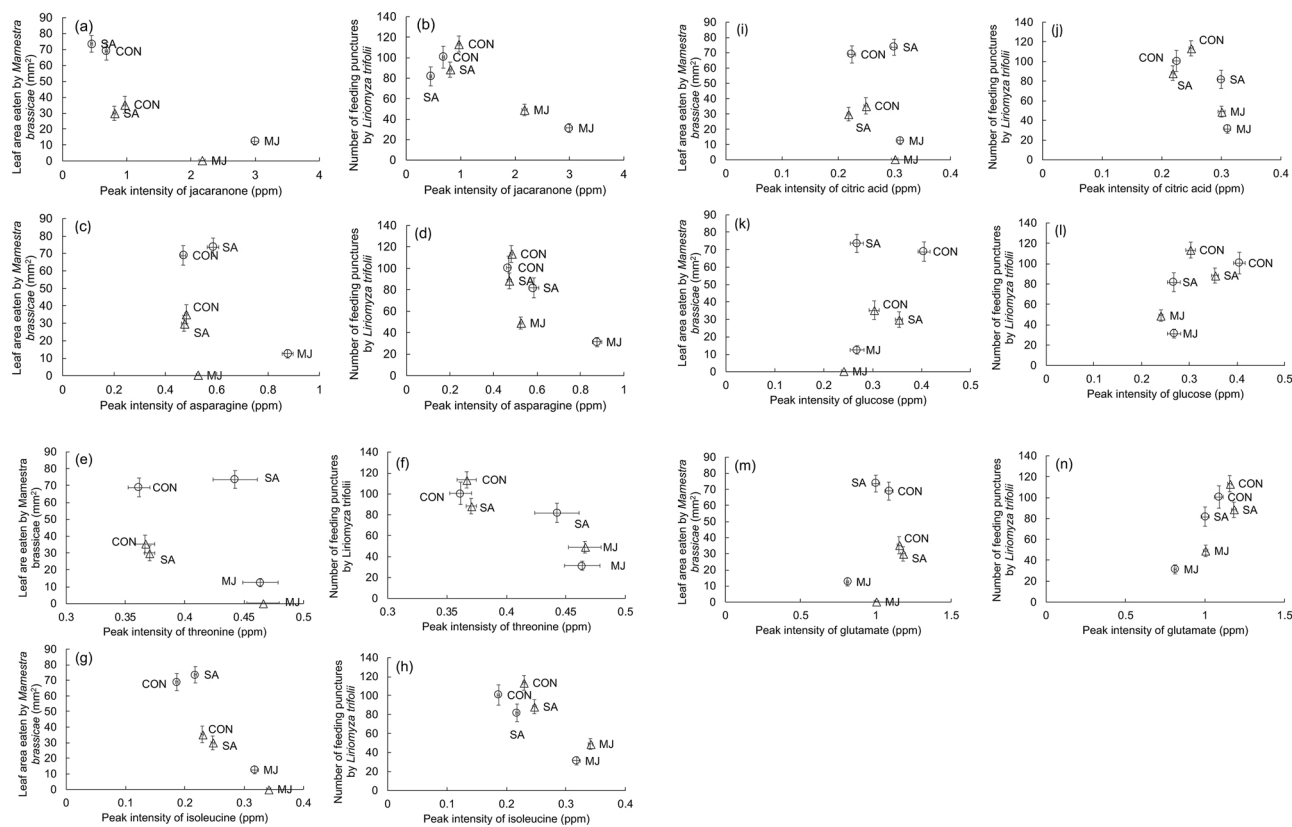


Fig. 4. Potential compounds affecting the feeding of caterpillar *Mamestra brassicae* and leafminer *Liriomyza trifolii* per treatment in *Jacobaea vulgaris* and *Jacobaea aquatica*. Triangles represent *J. vulgaris* and circles represent *J. aquatica*. Horizontal error bar represents the standard error of peak intensity of metabolites and vertical one represents the standard error of feeding damage of herbivores.

thrips (Fig. 5a–c), while glucose and 3,5-DCQA were identified as candidate compounds involved in plant susceptibility to thrips in *J. aquatica* (Fig. 5d,e).

4. Discussion

The metabolic profiles of both plant species showed similar shifts after application of phytohormones. The peak area of more NMR bins was affected by phytohormone treatments in *J. aquatica* than in *J. vulgaris*. The latter was especially true for the SA treatment, in which the NMR signals of 76 bins (Table 1) were affected significantly in *J. aquatica* while only seven bins were affected in *J. vulgaris*. Apparently, there was a strong species-specific effect in the strength of the response but not in the direction. In both plant species, MeJA treatment up-regulated the NMR signal in more bins while SA treatment down-regulated the NMR signal in more bins. The PCA plots showed a clear separation

Table 3

Summary of Spearman rank correlations between amount of silver damage of thrips and signal of 170 NMR bins in 102 F2 hybrids and the number of significant up-regulated and down-regulated NMR bins in the SA treatment compared to the control in *Jacobaea aquatica* and *Jacobaea vulgaris* after salicylic acid treatment.

Plant species	Variables	Positive ^a	Negative
F2 hybrids ^b	Number of significant correlations	17	25
SA treated <i>J. vulgaris</i>	Significant bin changes ^c	–	1↑
SA treated <i>J. aquatica</i>	Significant bin changes ^c	10↓, 2↑	5↑

^a ↑: bins with an up-regulated signal; ↓: bins with down-regulated signal.

^b In the NMR dataset of F2 hybrids, there were 170 bins between $\delta 7.68$ – $\delta 0.8$, but 7 bins had no signal ($\delta 4.96$, $\delta 4.92$, $\delta 4.88$, $\delta 4.72$, $\delta 4.68$, $\delta 4.64$ and $\delta 3.32$).

^c The number of NMR bins that are significant after adjusting for multiple comparisons by the Benjamini & Hochberg method.

between leaves from the control and MeJA treatments in both plant species. These results are consistent with other studies, which found that *Brassica rapa* and *Fagopyrum esculentum* plants treated by MeJA showed a distinct separation from control plants [34,35].

The number of bins with decreased NMR signals was 2–6 times higher than the number of bins with an increased NMR signal after SA treatment compared to the control (Table 1, *J. vulgaris*: 6/1; *J. aquatica*: 54/22). Metabolomic shifts upon exogenous SA application did not show a consistent pattern across different plant species. For example, SA application significantly induced the production of hyoscyamine and scopolamine in root cultures of *Brugamansia candida* [36], while the concentration of some of the major flavonoids decreased significantly after SA treatment in ginger (*Zingiber officinale* Roscoe) [37]. In our study the total concentration of PAs, the typical SMs in *Jacobaea* plants, were not affected by the SA treatment. As was the case after exogenous application of MeJA, the application of SA led to a more pronounced metabolic shift in *J. aquatica* compared to *J. vulgaris*.

The most important question from an ecological point of view is whether or not these metabolomic changes contribute to the plant's defense against pest and pathogens. Our previous study showed that MeJA changed the composition of pyrrolizidine alkaloids (PAs) in *Jacobaea* plants, leading to a shift from senecionine- to erucifoline-like PAs, which were much more toxic to *Spodoptera exigua* in *J. aquatica* [26]. In our current study, the leaf-chewing and leaf-mining herbivore caused less damage on MeJA treated leaves, indicating that herbivores with chewing and mining feeding modes could activate the JA pathway, which is consistent with the results found for other plant species [20].

Thrips is a piercing-sucking herbivore, which feeds on vascular tissue by inserting its mouthparts through the cells and causing minimum disruption. Attack by insects with this feeding mode often induces increased levels of SA in plants while some studies have shown that exogenous SA application increases endogenous levels of SA [38]. In our

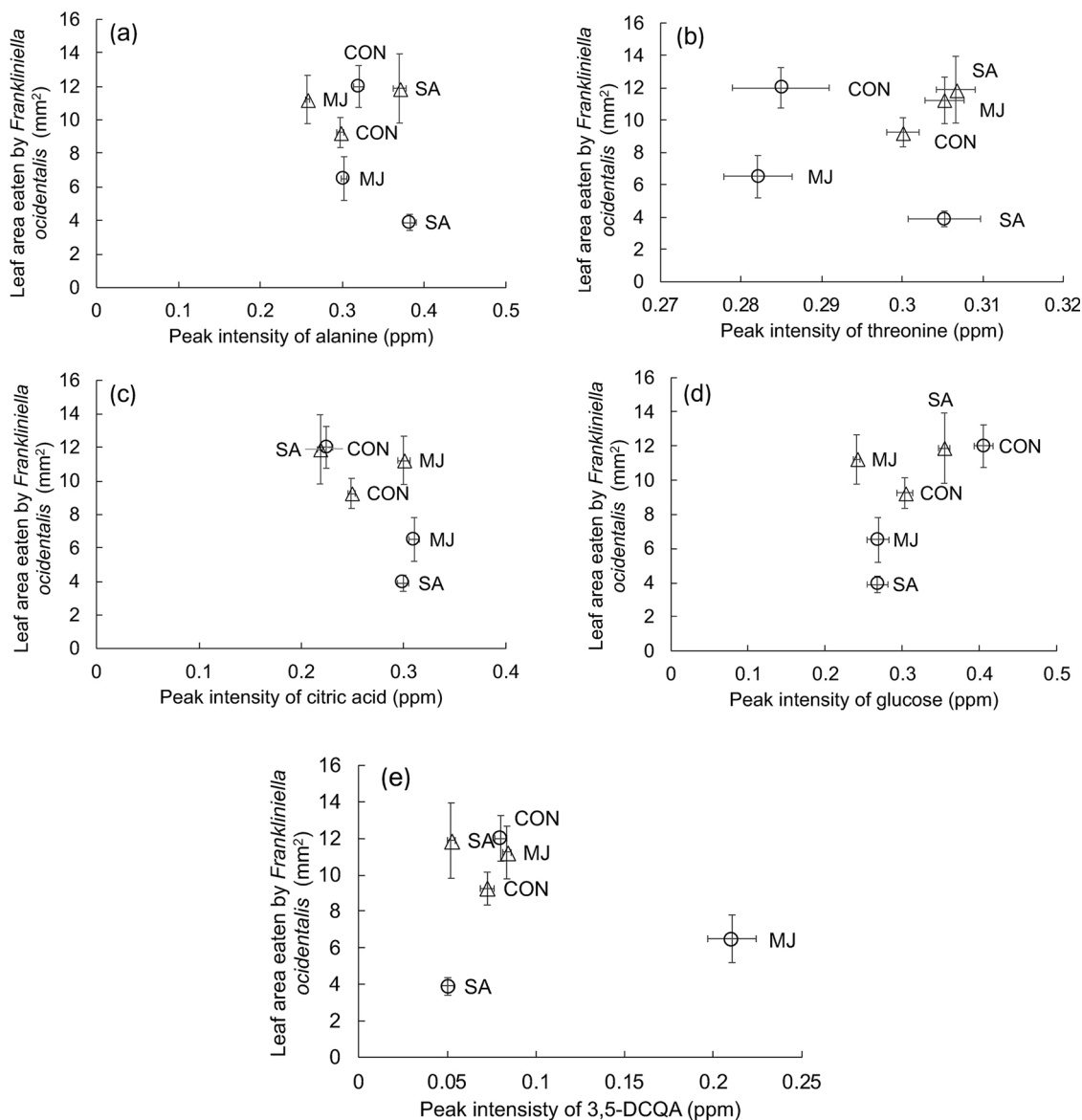


Fig. 5. Potential compounds affecting the feeding of thrips *Frankliniella occidentalis* per treatment in *Jacobaea vulgaris* and *Jacobaea aquatica*. Triangles represent *J. vulgaris* and circles represent *J. aquatica*. Horizontal error bar represents the standard error of peak intensity of metabolites and vertical one represents the standard error of feeding damage of herbivores.

study, the silver damage of thrips significantly decreased after SA application in *J. aquatica*, which is in line with the hypothesis that the feeding of sucking insects induces a SA-mediated defense pathway [8]. However, the resistance to thrips enhanced by JA treatment were found in other plant species, such as *Arabidopsis thaliana* [39], *Brassica rapa* [40] and tomato [21]. In our study, MeJA application indeed led to a reduced feeding of thrips in *J. aquatica* only, but it was not statistically significant.

Our studies showed that higher concentrations of jacaranone, asparagine, threonine, isoleucine and citric acid were potentially associated with lower feeding of chewing and leaf-mining herbivores, while higher concentrations of glucose and glutamate were associated with higher feeding of the two herbivores. Based on these combined results of the present experiment and the previous correlative studies [27,28], increased levels of alanine, threonine and citric acid were associated with less herbivory, while increased levels of glucose and 3,5-DCQA were associated with higher damage of thrips in *J. aquatica*. Therefore, threonine and citric acid were the common compounds that potentially reduced the feeding of the three herbivores, while the

glucose was the common compounds that potentially increased the feeding of the three herbivores. The other compounds showed herbivore-specific effect.

Pyrrolizidine alkaloids, the typical secondary metabolites in *Jacobaea* plants, are synthesized during amino acid metabolism in plants. Necic acids of PAs are mostly derived from L-valine, L-leucine, L-isoleucine and L-threonine [41]. In our study, threonine, isoleucine and valine increased after the phytohormone treatments although not all increases are significant (Fig. 3). Therefore, these amino acids syntheses may directly or indirectly control various aspects of *Jacobaea* plant defense.

Except for being involved in PA synthesis, isoleucine can be conjugated with JA to become JA-Ile [42]. A study demonstrated that exogenous MeJA activated defensive systems in plants boost themselves essentially by converting itself into JA and JA-Ile and initiating a signal transduction leading to volatile organic compound (VOC) emission and induction of endogenous JA-Ile and JA-Leu, which in turn caused further amplification of VOC emissions [43]. The susceptibility of *Manduca sexta* larvae was associated with reduced levels of JA-Ile in *Nicotiana*

attenuata [25]. In both *J. vulgaris* and *J. aquatica*, isoleucine significantly increased after MeJA application. Based on these studies, we speculate that the level of JA-Ile conjugates increased and consequently led to a higher plant resistance after *Jacobaea* plants were treated by MeJA, which to some extent was supported by the negative correlation between feeding damage and isoleucine in this study.

Citric acid is a weak organic acid and is an intermediate in the citric acid cycle. It can be used for plant protection against the most dangerous phytopathogens [44]. Citric acid is an important component of the stress response in *Leymus chinensis*, and exogenous application of 50 mg L⁻¹ citric acid played a positive role on stress tolerance [45]. The NMR signal of citric acid in our study increased significantly, indicating a positive response to the exogenous application of phytohormones mimicking herbivores' feeding.

Glucose was found to induce tachykinin-related peptide (TRP) secretion in silkworm, while other sugars, including sucrose, fructose, and myo-inositol, did not induce TRP secretion [46]. The TRPs are one of the largest families of neuropeptides. Glucose decreased after exogenous application of phytohormone in this study, which would reduce the induction of TRP secretion. As a consequence, the feeding of herbivores was suspected to be reduced.

Jacaranone has been isolated from several *Senecio* species [47–49]. Jacaranone and its analogues have been shown to have insecticidal activity against the housefly *Musca domestica* and can inhibit the growth of generalist herbivore *Spodoptera litura* [47]. Our study showed that jacaranone increased 2–3 folds after MeJA treatment while it caused a significant decrease of feeding damage of *M. brassicae* and *L. trifolii*, suggesting that jacaranone might be involved in resistance to the leaf-chewing and leaf-mining herbivores. Leiss et al. [31] observed a higher concentration of jacaranone in the young leaves of thrips-resistant *Jacobaea* plant, but the silver damage of thrips was not correlated with jacaranone concentration in this study. The anti-herbivore activity of jacaranone needs to be further tested with pure compounds.

Glutamate is a wound signal, and a plant injured on one leaf can alter other leaves to begin anticipatory defense response via glutamate receptor-like ion channels [50]. Glutamate also played a key role in plant defense against pathogens [51]. During the interactions with pathogen, the plant glutamate metabolism is altered strikingly in response to different pathogens in two opposing ways, either assisting the ongoing defense strategy to ultimately form an efficient resistance response or being exploited by the pathogen to promote and facilitate infection [51].

Alanine and asparagine are used in the synthesis of proteins. Asparagine is known for its key role in the biosynthesis of glycoproteins [52]. 3,5-DCQA is a phenolic acid with antioxidant and anti-inflammatory activities [53]. Additionally, based on the correlation analysis, the compound might play an herbivore-specific role in resistance/susceptibility, but not much is known about direct effects of these three compounds on herbivores.

5. Conclusions

The metabolites in the two *Jacobaea* species showed a similar response pattern: the MeJA treatment induced more metabolites to be upregulated while the SA treatment showed more metabolites that were down-regulated compared to the control. However, the up and down regulation was more pronounced in *J. aquatica*. The feeding of leaf-chewing (*M. brassicae*) and leaf-mining (*L. trifolii*) herbivores was significantly reduced in the MeJA treated leaves while the piercing-sucking herbivore (*F. occidentalis*) caused less damage to SA treated leaves of *J. aquatica* only. Among the 16 compounds identified, threonine and citric acid are associated with resistance and glucose is associated with susceptibility to the three herbivores tested. Jacaranone, asparagine and isoleucine are associated with resistance to the leaf-chewing *M. brassicae* and the leaf-mining *L. trifolii*, alanine is associated with resistance to piercing-

sucking *F. occidentalis*. Glutamate and 3,5-DCQA are associated with susceptibility in the leaf-chewing *M. brassicae* and the leaf-mining *L. trifolii*. These candidate amino acids function as the precursors of biosynthesis of SMS, or reduce the palatability of the plants. Because the results of the current study were based on correlation analyses, further investigations with pure compounds or transcriptome analysis are needed. Despite all this, our study clearly demonstrated that MeJA and SA can be used as elicitors to induce plant defenses, and not only SMS but also PMs are associated with plant resistance/susceptibility to the herbivores from different feeding modes.

Author contributions

Klaas Vrieling, Peter G. L. Klinkhamer and Xianqin Wei conceived and designed the research. Xianqin Wei performed the experiments. Hye Kyong Kim and Patrick P. J. Mulder did the chemical analysis. All the authors participated in the data analysis. Xianqin Wei wrote the manuscript. Klaas Vrieling, Peter G. L. Klinkhamer and Patrick P. J. Mulder revised the manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.plantsci.2020.110784>.

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