Chapter 5.

Cells specific metabolomics on F2 hybrids of *Jacobaea vulgaris* and *Jacobaea aquatica*

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ABSTRACT

Plants are complex organisms that consist of different cell types and each cell type contains its unique chemical composition to support its specific function. We therefore applied laser microdissection coupled with NMR metabolomics to study the distribution of primary and secondary metabolites in epidermis, palisade and spongy mesophyll leaf cells of *Jacobaea* plants. Two genotypes, of F2 hybrids of *Jacobaea vulgaris* and *Jacobaea aquatica,* genotype A resistant and genotype B susceptible to western flower thrips, with 3 replicates each, were used. For each cell type approximately 3000 cells were collected and analysed. The partial least square discriminant analysis (PLS-DA) showed a clear separation of the three different cell types for both genotypes. Regarding primary metabolites the epidermis cells showed lower levels of glucose, threonine and the organic acids malate and succinate but higher levels of cholin as well as of amino acid alanine in comparison with the two mesophyll cell types. With regard to secondary metabolites the epidermis cells contained lower concentrations of the PA jacobine *N*-oxide, which was concentrated in the palisade cells, but higher concentrations of the phenylpropaonid chlorogenic acid and the flavanoid kaempferol glucoside. Although both genotypes showed almost the same metabolite profiles over the three cell types, the resistant genotype contained significantly more fructose, threonine and jacobine *N*-oxide compared with the susceptible genotype. The epidermis as outer cell layer confers protection to the cells. Therefore, it was rich in metabolites ameliorating the negative effects of abiotic factors such as UV-light, drought, frost and salt and rich in plant defence compounds against general pathogens and herbivores. However, jacobine *N*-oxide compounds may also act as host finding cues for specialist herbivores. In order to solve this generalist-specialist dilemma the distribution of the defence compounds may adapt as in the case of the PA jacobine *N*-oxide, which did not accumulate in the epidermis but in the palisade mesophyll beneath. In contrast the mesophyll cells, containing chlorophyll, were generally rich in metabolites related to respiration and energy generation. As such our study emphasises the importance of studying the cell-specific distribution and function of metabolites in distinguished cell types.

Keywords: cell type specific metabolomics, chlorogenic acid, laser microdissection, *Jacobaea* sp., jacobine, NMR, pyrrolizidine alkaloids

INTRODUCTION

Plants, as complex organisms distribute specific functions to different types of organs and tissues. These distinct functions result from the integrated activity of individual cells (Schad et al., 2005). Around forty different cell types occur in plants (Martin et al., 2001) of which twelve are in the leaves alone (Nelson et al., 2008). Epidermal cells, as the outer leaf tissue, represent the outer barrier of the leaves with its environment. As such they are involved in gas exchange, water homeostasis, and plant protection (Gutiérrez-Alcalá et al., 2000). Hence, the internal leaf tissue is needed for photosynthesis. Despite this common function, a number of cell types such as the palisade and the spongy mesophyll as well as the bundle sheath cells can be distinguished (Langdale, 1998). These different cell types have their own specific biological functions that play different roles in plant growth and development (Murata et al., 2008). Therefore, each cell type contains its own unique chemical composition to support its specific function (Day et al., 2005; Martin et al., 2001). Studying the chemical composition of each cell type will help us to identify how different compounds are compartmentalised and to understand how and where these compounds are biosynthesised and what their functions in plant survival are.

Efforts to conduct cell specific studies have been started since a few decades ago. In the early phase, most of these studies mainly used *in-situ* methods such as enzyme histochemistry and RNA hybridization that rely on localization of an indicator in a cellular region. In such approach the cells remain in the context of the tissue (Outlaw and Zhang, 2001). Recently, at least two approaches are available for collecting specific cell types. The first is to sample single cells from living plant tissues using microcapillaries (Karrer et al., 1995). The second is the removal of the sample interest from the tissue context. Several protocols have been developed to remove specific cells, including protocols for the isolation of leaf surface cells like glandular trichomes that protrude on the leaf surface (Lange et al., 2000; Gang et al., 2002; Wagner et al., 2004) and isolation of epidermal cells by carborondum abrasion (CA) (Murata and de Luca, 2005). Among the different cell types of the leaf, epidermis cells have been studied more frequently. It was revealed that in most plant species, the epidermis cell plays specialised roles in biosynthesis and accumulation of a wide range of secondary metabolites (SMs), including flavanoids (Kutchan, 2005), terpenes (Dudareva et al., 2005), and alkaloids, (Murata and de Luca, 2005). The latter study was conducted using RNA isolation and gene expression analysis on *Catharanthus roseus* describing the spatial distribution of the intermediate compounds, vindoline and catharanthine, as members of the monoterpenoid indole alkaloids pathway (Murata and de Luca, 2005). These alkaloids are the components of the commercially important anticancer dimers, vinblastine and vincristine.

The most advanced technique in the single cell isolation is the use of laser microddissection (LMD). In this technique, the tissue is stabilised before the cells are dissected. Laser microdissection has been proven to be an effective technique to cut and collect single cell types. Firstly, LMD was applied to isolate cancer cells (Emmert-Buck et al., 1996) but has in the mean while been adapted to plant sciences for isolation of different cell types in proteomic studies (Banks et al., 1999) and gene expression studies (Nakazono et al., 2003). As such it has been used to determine the SMs content of specific plant cells. The stone cells of Norway Spruce, *Picea abies*, bark contained, next to lignin also stilbene astringin and dihydroflavonol dihydroxyquercetin 3'-*O*-β-D- glucopyranoside

(Li et al., 2007). Thus, the stone cells function as more than just repositories for lignin but may also be involved in chemical as well as physical defence against bark beetles and their associated microorganisms. Until now only a few studies used LMD for cell metabolomics. Application of LMD on cell specific metabolomics will allow us to detect the potential key metabolites in each cell type (Day et al., 2005). This approach is important especially if we do not have a prior knowledge about the compounds in the plant. For conventional metabolomics usually whole plant organs such as leaves, flowers, and roots are used mostly ignoring the different cell types contained in such organs. This leads to averaging and diluting the information of the different micro-metabolomes (Schad et al., 2005). An example of cell specific metabolomics comprises the comparison of metabolites in the vascular bundles with non-vascular cells in *Arabidopsis thaliana* (Schad et al., 2005). This study revealed a high accumulation of simple sugars such as galactose, fructose and glucose in the vascular bundles cells while disaccharide sucrose and TCA (threecarboxilic acid) cycle intermediates such as malic acid, isocitric acid and citric acid were higher in the leaves section without the vascular bundle cells. This study emphasises the effectiveness of this technique to identify the distribution of major small metabolites such as simple sugars, amino acids, etc. in the different type of cells.

Plants in the genus *Jacobaea* (Syn. *Senecio*, Asteraceae) represent an excellent study system with respect to the evolutionary ecology and biosynthesis of secondary defence metabolites (Pelser et al., 2005; Hartmann and Ober, 2000). These plants are known to constitutively synthesise pyrrolizidine alkaloids (PAs) (Hartmann and Zimmer, 1986). Aside from that, several SMs known as defence compounds such as chlorogenic acid (CGA), kaempherol glucoside and jacaranone have been reported to be present in these plants. Plants are attacked by many types of herbivorous insects. However, these differ in the specific plant tissue they will attack. For example, caterpillars chew on different plant organs as a whole, leafminers chew through the leaf mesophyll, thrips and mites suck up the content of epidermis cells and aphids feed on the phloem. Indeed, in a previous study, we reported a tissue specific distribution of the defence compounds chlorogenic acid (CGA) and PAs in *Jacobaea* plants (Nuringtyas et al., 2012). While CGA was highly accumulated in the epidermis, the PAs were concentrated s in the mesophyll. In regards with specialist herbivores, this high accumulation of PAs in the mesophyll tissues may serve as a defensive strategy to reduce the apparent of *Jacobaea* plants to specialist. The high accumulation of CGA in the epidermis may also serve as first line defence against certain types of insects which only attack epidermis such as thrips and mites. Thus, for plant host resistance study, this result emphasises that using leaf-level analysis one may fail to identify SMs with a specific compartmentation since the metabolites may be diluted by the other metabolites present in higher concentration in the leaves in other cells types. Considering the importance of specific compartmentation of defence compounds, in this current study, we chose two different genotypes: a thrips resistant and a susceptible one which may represent the variation within *Jacobaea* plants.

In this study, we applied the LMD technique to isolate the different cell types of the leaves: epidermis, palisade- and spongy-mesophyll cell followed by NMR metabolomics. We specifically addressed the following questions: Is the distribution of metabolites cell-specific, i.e. is the distribution of metabolites different between epidermis, palisade and spongy mesopyll cells? If so, which metabolites contribute to this difference? Are the differences observed common to both genotypes?

MATERIALS AND METHODS

Plant Materials. F2 hybrids of *Jacobaea vulgaris* Gaertn and *Jacobaea aquatica* G. Gaertn, B. Mey and Schreb were used. We chose two genotypes, A and B, as representatives of intra-species variation in *Jacobaea* plants. These genotypes have been used in a previous study on host plant resistance to western flower thrips (Cheng et al., 2011), in which genotype A was resistant and genotype B susceptible. The genotypes are maintained in the department tissue culture collection. We used three replicates for each genotype giving a total of six plants. The tissue culture plants were transplanted to pots (11 cm diameter) filled with a 1:1 mixture of dune sand and potting soil. The plants were maintained in a growth chamber (16:8 L:D, 20:15±2 °C) for four weeks. Each day two leaves of 2-3 cm length were harvested for cell collection over a period of five days.

Tissue Preparation. The harvested leaves were microwaved for 10 s at 400 W to deactivate enzymes. A 5x7 mm^2 leaf slice was dissected using a scalpel. In order to obtain sufficient rigidity when freezing the tissue using Jung tissue freezing medium (Leica Microsystem, Nussloch, Germany) the slice was cut across the leaf midrib. After embedding the slice in the freezing medium, it was frozen in liquid nitrogen and immediately cut using a cryostat microtome (Leica CM1850, Bensheim, Germany) adjusted to -20°C. Serial sections of 30µm thickness were cut and mounted directly on a microscopic glass slide 76x26 mm ((ROTH, Germany). Each slide consisted of 20 sections.

Laser Microdisection (LMD). Three types of cells were collected from each plant: epidermis, palisade- and spongy-mesophyll cells, amounting to eighteen samples in total. Collection of a sufficient amount of cells for NMR analysis took about 5 days per plant. Cells were collected separately per day and pooled before NMR analysis. Collections of cells of the two respective genotypes were alternated weekly. The serial sections were used in a Leica LMD6000 system for cell collection. Cutting was performed in the visible light mode at 40x magnification. While cutting, the number of cells collected was counted. Each cell type was collected separately in the cap of a 0.5 ml microcentrifuge tube. The caps were filled with pure methanol to ensure that the cells were immediately extracted in the solvent. A preliminary study showed that CGA, due to laser heating, started to degrade after 1.5 h and was almost totally degraded after 3 h during LMD. Therefore, the cell collection per slide was limited to 1.5 h. At the end of dissection, the tube with samples was carefully closed and the tubes were centrifugated at 12,000 rpm, 4°C for 20 min. For ^{1}H NMR analysis about 0.4 mg material, equivalent to about 3000 cells was needed. Therefore, the tubes were weighed after centrifugation. The supernatant was collected and transferred to fresh microtubes to be dried under a stream of nitrogen and kept at 4°C until ^{1}H NMR analysis. The daily cell collections were pooled per cell type and genotype by adding 50 µl methanol. The samples were centrifugated at 12,000 rpm, 4°C for 20 min. The methanolic extract was transferred to a fresh microtube and samples were dried under a stream of nitrogen.

Metabolomics.

Sample preparation for ^{1}HNMR analysis. The eighteen dried extracts of epidermis, palisade- and spongy-mesophyll cells were subjected to NMR metabolomics. For NMR analysis 100 µl of freshly opened methanol-d_4 (99.96% Deutero GmbH, Germany) were added. The methanol-*d4* contained 0.17 mmol of 2,4,6 Trichloronitrobenzene (TCNB) as internal standard. The mixture was vortexed

at room temperature for 30 s and ultrasonicated for 3 min. Subsequently, the mixtures were centrifugated for 5 min at 12,000 rpm, 4°C for 20 min. An aliquot of 75 µl of the supernatant was transferred to a capillary NMR tube.

NMR analysis.^{1}H NMR spectra were recorded with a Bruker Avance 500 NMR spectrometer equipped with a cryogenic TXI probehead (Bruker, Karlsruhe, Germany) at 30ºC. Each ^{1}H NMR spectrum consisted of 1054 scans requiring 3 h acquisition time.

Quantification. For the quantification of identified metabolites using NMR spectroscopy, the peak area of selected proton signals belonging to the target compounds, and the peak area of IS, were integrated manually for all the samples.

The following equation was applied for the calculations (van Beek et al., 2007):

$$C = \left(\frac{\text{Integral (Target)}}{\text{Integral (IS)}} \times \frac{\text{* MW (Target)}}{\text{**MW (IS)}} \times \text{Weight (IS)} \right) / \text{number of cells}$$

Target = signal of the target compound,
IS = signal of internal standard,
* MW (Target) = molecular mass/ number of protons involved in the target signal,
**MW (IS) = molecular mass / number of protons involved in the internal standard (IS) signal,
C = concentration in one cell, Weight is in micrograms. The concentration of TCNB in each NMR tube was fixed as 0.17 mmol.

Metabolite concentration was expressed in mol per cell. The total concentration of each metabolite per leaf was calculated by adding the concentrations of the respective three cell types.

Data reduction and quantification of ^{1}H NMR data. Spectral intensities were scaled to total intensity and reduced to integrated regions of equal width (0.04 ppm) from d 0.4–10.0. The regions of d 4.75–4.93 and d 3.28–3.34 were excluded from the analysis due to the residual signals of water and methanol. Bucketing was performed using AMIX (Bruker) with scaling on total intensity. Partial least square-discriminant analysis (PLS-DA) was performed with the SIMCA-P software (v. 12.0. Umetrics, Umeå. Sweden). The scaling method for both analyses was uv. The PLS-DA model was validated using the permutation method through 20 applications and CV-ANOVA, which are default validation tools in the software. Differences in concentration of the identified compounds between different cells types were analysed using ANOVA with cell type nested within genotypes. Statistic analysis was performed using SPSS statistic 19 (Chicago, IL, USA).

RESULTS

Identification of Metabolites. ^{1}H NMR analysis of epidermis, palisade, and spongy cell extracts allowed the identification of different metabolites including amino acids, organic acids, carbohydrates, phenylpropanoids and PAs (Table 1). Identification of metabolites was based on NMR spectra of known compounds acquired in previous studies on *Jacobaea* plants (Leiss et al., 2009a; Nuringtyas et al., 2012) and from comparison with our in house NMR spectra database (Kim et al., 2010).

Table 1. 1H chemical shifts (d) and coupling constants (Hz) in epidermis, palisade and spongy cell extracts of F2 hybrids of *Jacobaea* leaves identified by 1D NMR spectra in MeOH-d_4

No	Compounds	Chemical shifts (ppm) and coupling constants (Hz)
1	Alanine	d 1.46 (H-3, d, J = 7.2 Hz)
2	Chlorogenic acid (5-*O*-caffeoyl quinic acid)	d 5.42 (H-5, ddd, J = 10,8 Hz, 9.8 Hz, 5.6 Hz), d 6.28(H-8', d, J = 15.9 Hz), d 6.78 (H-5', d, J = 8.62 Hz), d 6.95 (H-6, dd, J = 8.21 Hz, 1.9 Hz), d 7.05 (H-2', d, J = 1.9 Hz), d 7.57 (H7', d, J = 15.9 Hz),
3	Choline	d 3,22 (s)
4	Fructose	d 4.03 (H-1, d, J = 3.5 Hz);
5	Feruloyl quinic acid	d 5.57 (H-3, dt, J = 8.0 Hz, 3.1 Hz), d 6.39 (H-8', d, J = 15.9 Hz), d 7.62 (H-7', d, J = 15.9 Hz)
6	Glutamine	d 2.32 (H-3, m), d 2.05 (H-4, m)
7	Glucose	d 4.48 (H-β, d, J = 7,9 Hz), d 5.11 (H-1α, d, J = 3,85 Hz)
8	Inositol	d 3.15 (H-5, t, J = 9.27 Hz), d 3.43 (H-1, H-3, dd, J = 2.79 Hz, 9.78 Hz), d 3.96 (H-2, t, J = 2.67 Hz)
9	Jacobine *N*-oxide	d 6,25 (H-2, brs), d 4.74 (H-3a, dd, J = 6.74Hz, 14 Hz), d 5.55 (H-9a, d, J = 11.9 Hz), d 4.01 (H-9b, d, 11.9 Hz), d 5.20 (H-7, t, J = 5.0 Hz), d 3.99 (H-6a, dd, J= 14.2 Hz, 5.8 Hz) d 1.34 (H-18, s) , d 1.15 (H-19a, d, J = 6.23 Hz), d 3.01(H-20, d, J = 5.4 Hz) , d 1.20 (H-21a, d, J = 5.39Hz).
10	Kaempferol glucoside	d 7.18 (H-3 and H-5, d, J = 8 Hz), d 7.38 (H-2 and H-6, d, J = 8 Hz)
11	Malate	d 4.28(H-2, dd, J = 9.8 Hz, 3.3 Hz), d 2.38(H-3a, dd, J = 16.3 Hz, 6.8 Hz), d 2.68(H-3b, dd, J = 15.32 Hz, 3.2 Hz),
12	Succinate	d 2.52 (s)
13	Sucrose	d 5.39 (H-1, d, J = 3.8 Hz), d 4.13 (H-1', d, J = 8.5 Hz)
14	Threonine	d 1.32 (H-5, d, J = 6.6 Hz)
15	Fatty acid	0.98 (H-v, t, J = 7.5), 1.31 (CH3, brs), 1.6 (m), 2.02 (m), 2.35 (m)

The ^{1}H NMR spectra obtained were divided into three regions representing the aromatic, carbohydrate and amino acid regions at d 7.8 –5.5, d 5.5 – 3.0 and d 3.0 –1.0, respectively (Fig. 1). In the aromatic region phenylpropanoids signals including CGA and 5-*O*-feruloyl quinic acid (ferulic acid, FQA) were observed. Aside from that, we could identify kaempferol glucoside based on its characteristic doublet signals at d 7.38 (*J* = 8.0 Hz) and d 7.18 (*J* = 8.0 Hz). The identification of this metabolite was confirmed by comparison with our in house NMR library as well by reported data (Leiss et al., 2009b). In the carbohydrate region, the protons of sucrose, glucose, raffinose, stachyose and fructose were identified. In addition, signals at d 3.15 (t, *J* = 9.3 Hz), d 3.43 (dd, *J* = 9.8 Hz, 2.8 Hz), and d 3.96 (t, *J* = 2.7 Hz) were assigned to inositol. Another sugar alcohol identified was mannitol with its characteristic doublet signal at d 3.80 (*J* = 3.0 Hz). In the amino acid region, glutamine, alanine and threonine were identified. Signals of fatty acids were detected at d0.98 (t, *J* = 7.5 Hz) and d 1.31 (brs). Jacobine *N*-oxide, belonging to the PAs, was identified based on the broad singlet of H-2 at d 6.25, characteristic of PAs, and the presence of doublet signals at d 1.20 (*J* = 5.4Hz) and d 1.15 (*J* = 6.2 Hz) in combination with a singlet at d 1.34 (Fig. 2). This identification was confirmed by comparing the sample spectra with a jacobine *N*-oxide standard. The methyl signals were not in the crowded area thus they were used for subsequent PA quantification.

Partial Least Square-Discriminant Analysis of ^{1}H-NMR Data. *Partial Least Square-Discriminant Analysis* score plot showed a clear separation of the three different cell types for both genotypes (Fig 3a and b). In both cases, the epidermis cells were located in the positive quadrant of PC1 while the palisade mesophyll cells were in the negative quadrant. The spongy mesophyll cells were located in the middle between epidermis and palisade mesophyll cells. The PLS-DA score plot of genotype A explained a total variation of 51.7% while the score plot of genotype B explained 66.2%. The permutation test of both genotype A and genotype B PLS-DA showed that the R^2 and Q^2 of the permuted Y vectors were lower than the original ones (Fig. 3c & d). The CV-ANOVA of both PLS-DA gave significant results (A: F = 7.01, df = 4, P = 0.049. B: F = 14.03, df = 4, P = 0.0012). Although the distribution pattern of the metabolites over the three leaf cell-types was the same in both genotypes, significant differences in the amount of specific metabolites between genotype A and B were observed. The PLS-DA score plot showed a clear separation of genotype A and B cell extracts (Fig. 4a). All cell extracts of genotype A were located in the negative quadrant of PC 1 while those of the B were in the positive quadrant. PC1 explained 33.6% of the variation while PC2 explained 19.0%, amounting to a total of 52.6% of explained variation. The model resulted in a variance R^2 of 0.652 and a predictive ability Q^2 of 0.526. The permutation test showed that the Q^2 of the permuted Y vectors was lower than the original one (Fig. 4b). Additional cross validation using CV-ANOVA gave a significant result (F = 8.83, df = 17, P = 0.005). The loading plot of the PLS-DA showed that the separation of the genotype A was highly affected by the primary metabolites fructose, succinate and threonine as well by the secondary metabolites jacobine *N*-oxide, CGA, FQA, and kaempferol glucoside, (Fig. 4c).

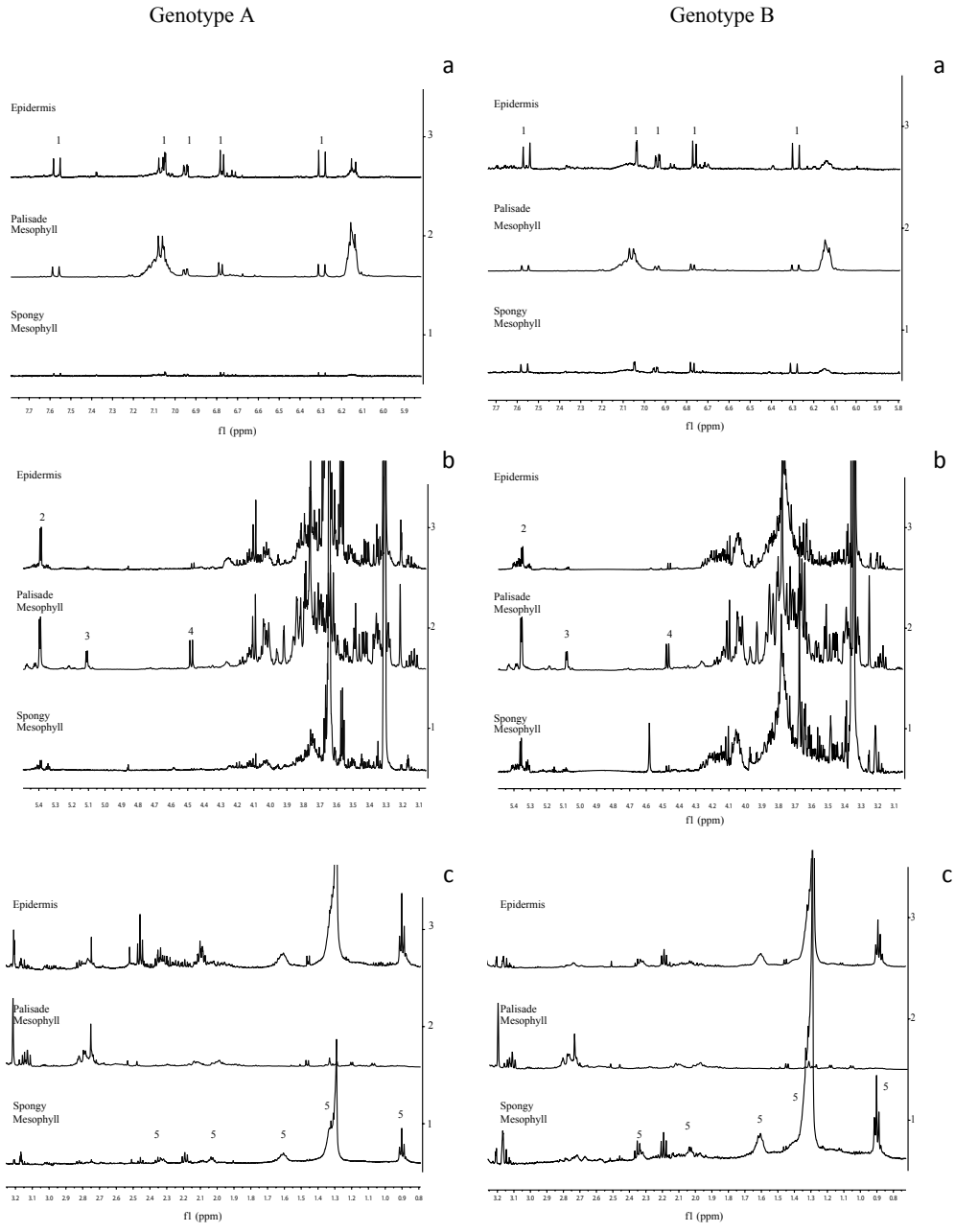

Fig 1. ^{1}H NMR spectra of the aromatic (A), sugar (B) and methyl (C) region for methanol extracts of epidermis, palisade- and spongy mesophyll leaf cells of genotypes A and B of F2 *Jacobaea* hybrids. Metabolites are labeled as chlorogenic acid (1), sucrose (2), α glucose (3), β glucose (4) and fatty acids (5).

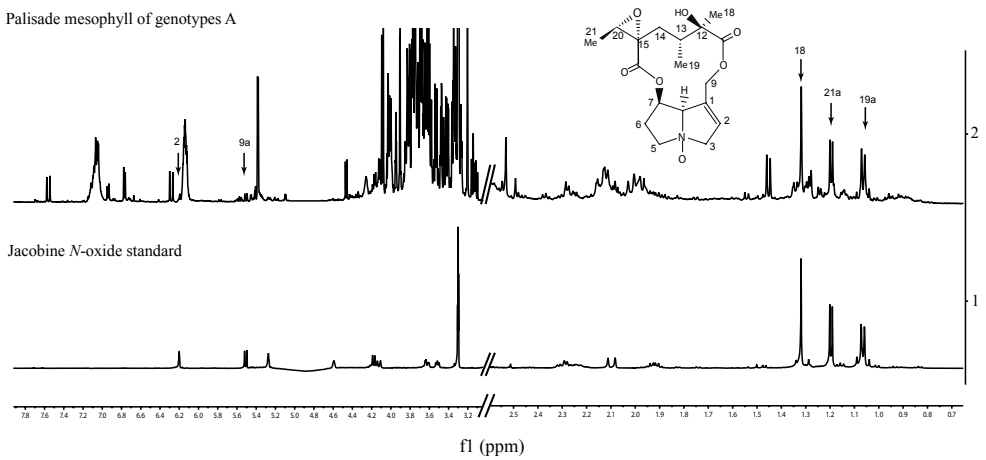

Fig 2. [1]H NMR spectra for methanol extracts of palisade mesophyll leaf cells of genotypes A of the F2 *Jacobaea* hybrids compared to the jacobine *N*-oxide standard.

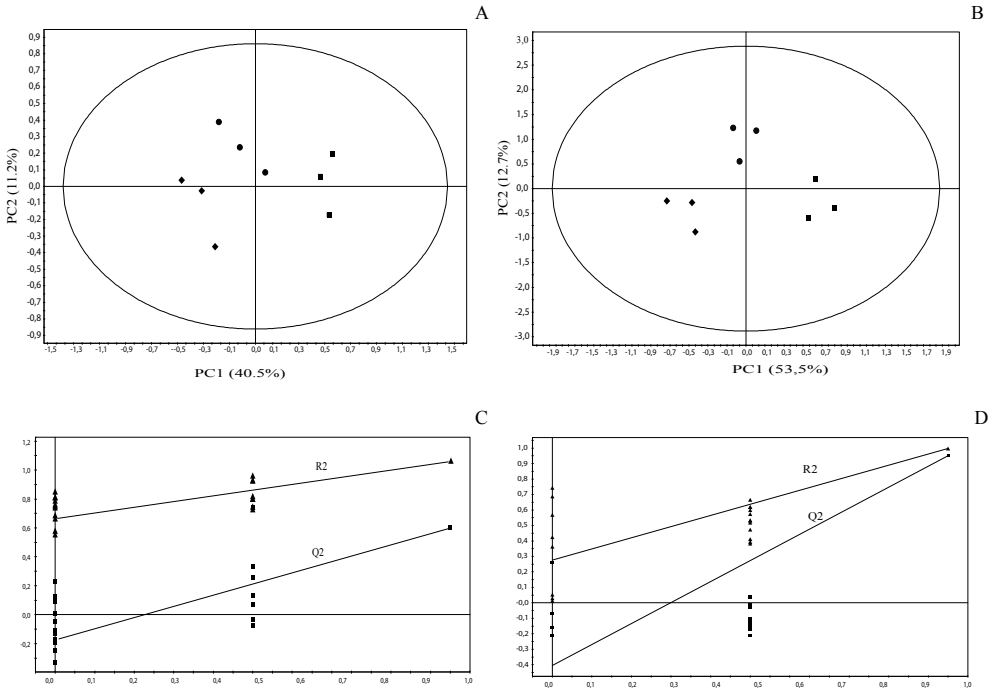

Fig 3. PLS-DA score plots for different cell types: epidermis (■), palisade mesophyll (♦) and spongy mesophyll (●) based on [1]H NMR signals of genotypes A (A) and genotypes B (B) of F2 *Jacobaea* hybrids and permutation validation (20 permutations with three components) of the PLS-DA plot of A (C) and B (D) genotypes.

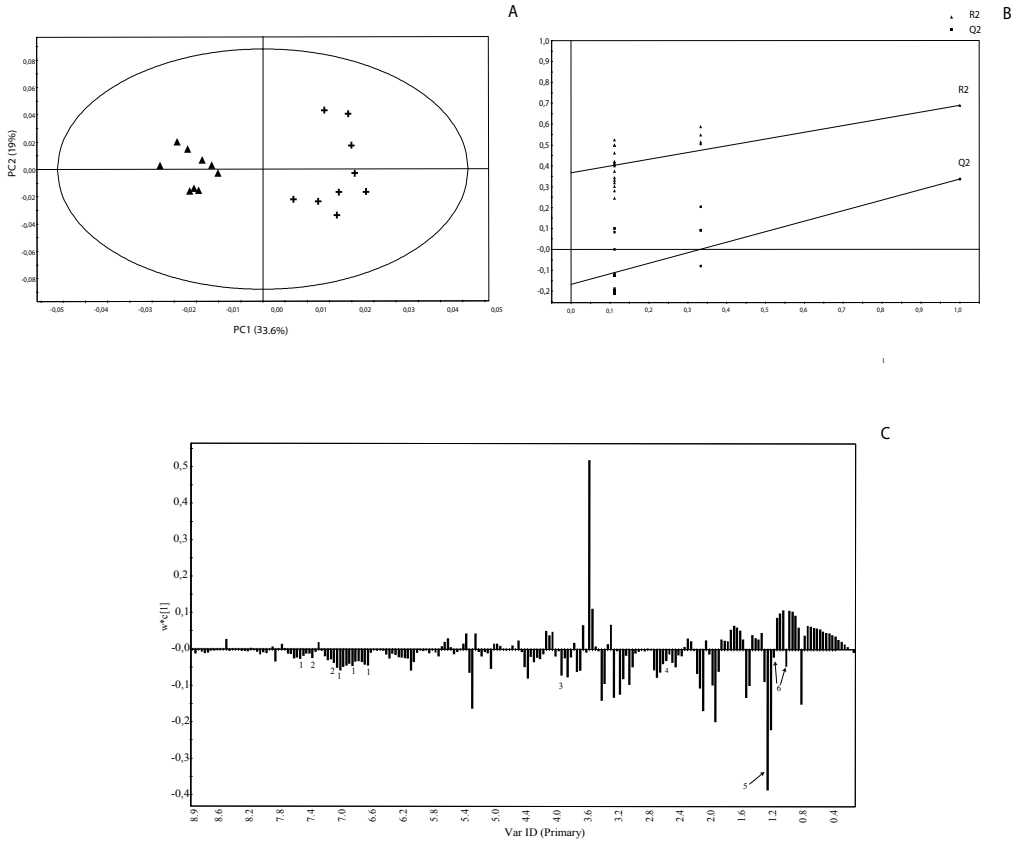

Fig 4. Score (A) loading plot (B) and permutation validation (20 permutations with three components) plot (C) of PLS-DA based on ^{1}H NMR signals of genotypes A (▲) and genotypes B (+) of F2 *Jacobaea* hybrids. Metabolites are labeled as chlorogenic acid (1), kaempferol glucoside (2), fructose (3), succinate (4), threonine (5) and jacobine *N*-oxide (6).

Quantification of Metabolites. Plant cell types differed significantly in primary and secondary metabolites as depicted in Figs. 5 and 6. In regard to the primary metabolites the epidermis cells were low in both alpha glucose ($F = 4.06$, $df = 2$, $P < 0.04$) and beta glucose ($F = 14.08$, $df = 2$, $P < 0.0001$) levels and threonine ($F = 4.52$, $df = 2$, $P = 0.04$) compared to the spongy mesophyll cells. With regard to the organic acids, epidermis contained lower levels of malate ($F = 11.17$, $df = 2$, $P = 0.006$) compared to palisade- and spongy- mesophyll cells and succinate ($F = 11.17$, $df = 2$, $P = 0.001$) compared to the palisade mesophyll cells. In contrast they were rich in cholin, a quaternary ammonium salt, ($F = 38.32$, $df = 2$, $P < 0.0001$) and amino acid alanine ($F = 4.59$, $df = 2$, $P = 0.029$) compared to both palisade- and spongy- mesophyll cells. Looking at the SMs the epidermis cells contained lower concentrations of the PA jacobine *N*-oxide ($F = 14.66$, $df = 2$, $P < 0.0001$), which was concentrated in the palisade cells, but higher concentrations of the phenylpropanoid CGA ($F = 19.65$, $df = 2$, $P < 0.0001$) compared to the palisade- and spongy-mesophyll. Similar distribution patterns with CGA was also observed the flavanoid kaempferol glucoside ($F = 13.01$, $df = 2$, $P = 0.001$) that is higher level in epidermis cell compared to the spongy mesophyll cells.

Significant quantitative differences in metabolites between gentoypes occured in the primary metabolites fructose and threonine as well as in the SMs jacobine *N*-oxide. Genotype A contained one-fourth more of fructose (F = 438.84, df = 1, P = 0.002), double as much threonine (F = 232.04, df = 1, P = 0.030) and two folds of the PA, jacobine *N*-oxide (F = 32.628, df = 1, P = 0.031) compared to genotype B. In the PLS-DA loadings plots, succinate was identified as important factor for the separation between the two genotypes. However in the quantitative analysis, it did not show any difference. This may due to the presence of other signals which may influence the PLS-DA analysis.

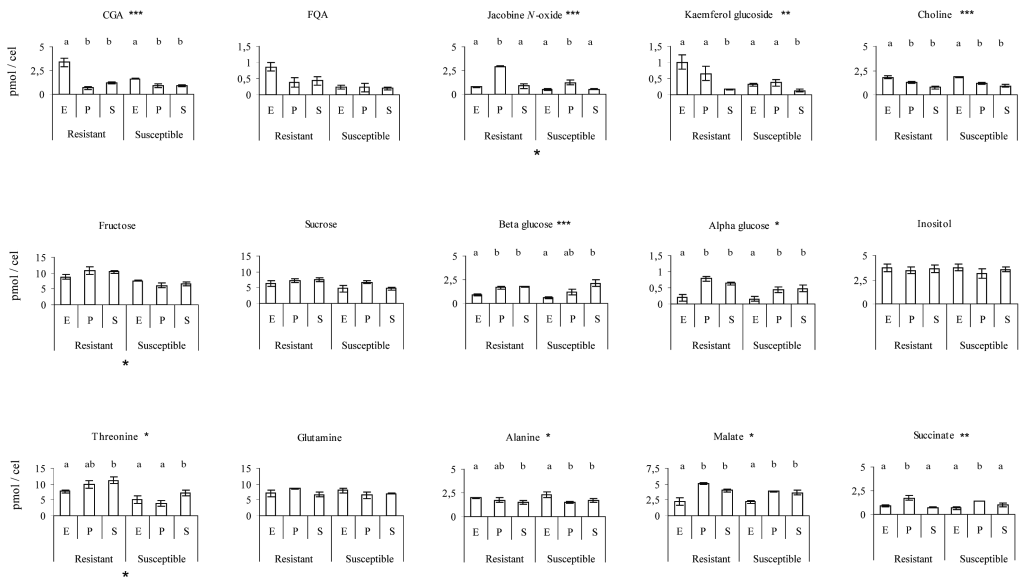

Fig 5. Concentration of metabolites in epidermis (E), palisade- (P) and spongy mesophyll (S) leaf cells of genotypes A and B of F2 *Jacobaea* hybrids. Means of three replicates and the standard error are presented. Data were analyzed by nested ANOVA with cell type nested within genotypes. Significant differences between A and B genotypes are indicated at the base of the graphs, whereas significant differences between cell types are indicated at the top of the graph whereby * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$.

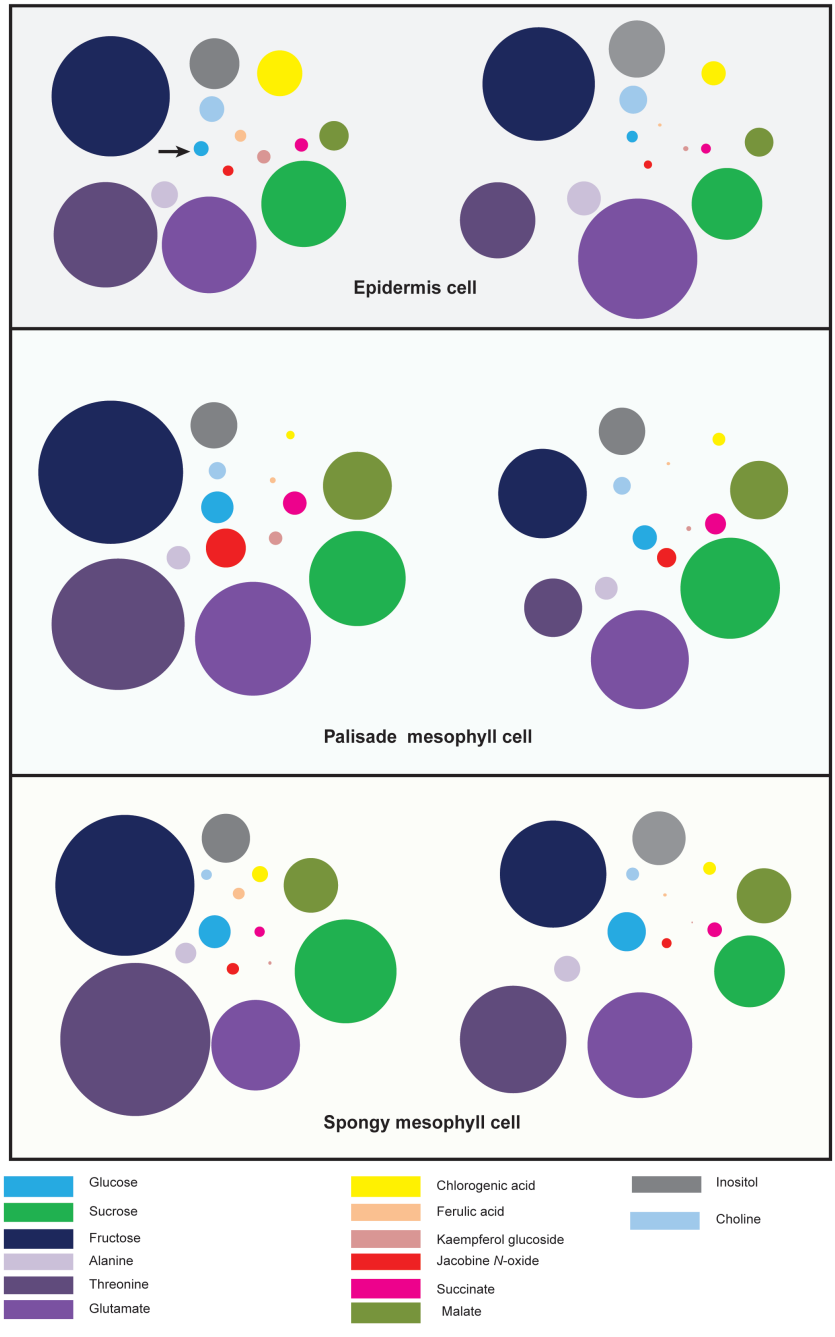

Fig 6. The distribution of metabolites in epidermis (E), palisade- (P) and spongy mesophyll (S) leaf cells of F2 *Jacobaea* hybrids. The area of the circles represent the ratio of respective metabolites levels compared to glucose levels in epidermis of the genotype A. → indicated the glucose which was used to determine the ratio.

DISCUSSION

Cell-specific metabolomics of epidermis, palisade- and spongy- mesophyll cells showed that metabolites contained in these three cell types were cell specific. A similar pattern of cell-specific distribution of primary and secondary metabolites was observed for both genotypes tested, indicating that such a specific distribution may be of general occurrence.

Regarding primary metabolites the epidermis cells showed lower levels of glucose, threonine and the organic acids malate and succinate but higher levels of cholin as well as of the amino acids alanine in comparison with the two mesophyll cell types. The epidermis cell in contrast to the mesophyll cell is free of chlorophyll. Chlorophyll enables photosynthesis of which glucose is the primary product. In our earlier study (Nuringtyas et al., 2012), we also reported low levels of glucose in the epidermal leaf tissue. Similarly, malate, as a key metabolite, is involved in respiration and energy generation through photosynthesis (Martinoia and Rentsch, 1994). Cholin, as an intracellular salt, plays a role in protecting plants against unfavourable conditions such as salt, drought or cold stress (Sakamoto and Murata, 2002). Higher accumulation of alanine may serve as a source of beta-alanine and beta-alanine betain which was known as one of the most effective osmoprotectant (Rathinasabapathi et al., 2000). The amino acid threonine has been indicated as a precursor of the necid acid of PAs (Stirling et al., 1997). As for threonine also the levels of PAs, as SMs were increased in the mesophyll cells in contrast to the epidermis cell. The same finding but then on the tissue level (Nuringtyas et al., 2012) confirms our result. Thus, SMs showed a cell-specific distribution pattern. The epidermis cells contained lower concentrations of the PA Jacobine *N*-oxide, which was concentrated in the palisade cells, but higher concentrations of the phenylpropaonid CGA and the flavanoid kaempferol glucoside.

In the genus *Jacobaea* PAs are associated with plant defence against generalist herbivores as described in the review by Macel (2011). Particularly the jacobine type PAs, including jacobine *N*-oxide, are involved in host plant resistance to Western Flower thrips (Leiss et al., 2009a, Cheng et al., 2011). However, Macel and Klinkhamer (2010) indicated that jacobine has a positive effect on specialist feeding. In a field study they observed a positive correlation between jacobine concentration and damage of the cinnabar moth (*Tyria jacobaeae*), the thrips *Haplothrips senecionis* and the Rust fungus *Puccinia dioicae,* respectively. We showed that jacobine *N*-oxide was highly accumulated in the cells of the palisade mesophyll, which may constitute a strategy to deal with the generalist-specialist dilemma. Jacobine serves as a plant defence compound against generalists but at the same time attracts specialists. The cinnabar moth can use PAs as an oviposition cue (Macel and Vrieling, 2003; Cheng et al., 2013). Placing jacobine in the palisade cells may ensure plant defence against the chewing larvae but prevent the use of jacobine as a host recognition cue for oviposition on the leave surface. Similarly, *P. diociae*, as an obligate biotroph pathoghen, may be inhibited to use jacobine on the leave surface for host recognition. Thrips, which are piercing-sucking insects, commence feeding with probing of the epidermis followed by ingestion of the sub-epidermal cells (Harrewijn et al., 1996; Kindt et al., 2003). High concentrations of jacobine in the sub-epidermal palisade cells may thus prevent the specialist thrips *H. senecionis* from recognising its host and at the same time prevent from feeding by the generalist thrips *F. occidentalis*. The accumulation of jacobine in the palisade cells confirms our

earlier finding reporting a high concentration of PAs, including jacobine, jaconine and senecionine, as both, the free base and *N*-oxide forms, in the mesophyll tissue of *Jacobaea* plants. However, in that study the two different mesophyll cell-types could not be distinguished. Due to the micro-metabolomic approach, looking at single cells, the amount of material analysed in the current study allowed to detect only the most abundant PA: jacobine *N*-oxide.

In line with our previous study (Nuringtyas et al., 2012), CGA was highly accumulated in the epidermis cells. A high concentration of phenylpropanoids, including CGA, in the epidermis of *Arabidopsis* mutants was associated with its function as a UV-B protector in plants (Bharti and Khurana, 1997). Treatment of wild tobacco (*Nicotiana attenuata*) plants with UV-B radiation increased the level of CGA and subsequently decreased the amount of leaf damage caused by the thrips *F. occidentalis* (Demkura et al., 2010). Accumulation of CGA has been reported to be related to thrips resistance in chrysanthemum (Leiss et al., 2009b). From the biosynthesis point of view, the higher amounts of CGA are based on the activity of phenylalanine ammonia-lyase, the key enzyme of CGA biosynthesis which is situated in the epidermis (Kojima and Conn, 1982).

Another secondary metabolite associated with plant defence, kaempferol glucoside, was accumulated in the epidermis and palisade cells. Kaempferol glucoside has been identified in *Jacobaea* hybrids earlier (Leiss et al., 2009; Kirk et al., 2005). Kaempferol is known to confer a deterrent effect on onion thrips, *Thrips palmi*, (Wu et al., 2007), generalist caterpillars (Onyilagha et al., 2004) and aphids (Lattanzio et al., 2000). In a study on tissue localization of phenolics, high concentrations of kaempferol glucosides were detected in the epidermis of Broad Bean, *Vicia fab*a, (Hutlzer et al., 1998). Schnitzler et al. (1996) reported the accumulation of kaempferol glucosides, as UV-B pigments, in the epidermis of Scots Pine, *Pinus sylvestris*.

While the above patterns of cell-specific metabolites were the same for both genotypes tested, the thrips resistant genotype A contained higher amounts of fructose, threonine and jacobine *N*-oxide compared to the susceptible genotype B. Carbohydrates, especially sucrose and fructose are strong feeding stimulants for herbivorous insects (Bernays and Simpson, 1982). Naturally, they are also important nutrients for the insects to synthesise body tissue and to serve as an energy source (Schoonhoven et al., 2008). Threonine, as described above functions as a pre-cursor for the necic acid of PAs (Stirling et al., 1997) and may thus serve to supply the synthesis of jacobine. Higher amounts of jacobine *N*-oxide in *Jacobaea* plants resistant to *F. occidentalis*, in contrast to susceptible ones have been reported by Leiss et al. (2009a) and Cheng et al. (2011). The plant defence compounds CGA and kaempferol glucoside were both somewhat increased in the resistant genotype but this was not significantly different.

CONCLUSION

Cell-specific metabolomics of epidermis, palisade- and spongy- mesophyll cells showed that metabolites contained in these three cell types were cell-specific for both genotypes indicating that this may be a general pattern. The epidermis as outer cell layer confers protection to the cells. Therefore, it was rich in metabolites ameliorating the negative effects of abiotic factors such as UV-light, drought, frost and salt and rich in plant defence compounds against general pathogens and herbivores. However, the later compounds may also act as host finding cues for specialist herbivores. In order to solve this generalist-specialist dilemma the distribution of the defence compounds may adapt as in the case of the PA jacobine *N*-oxide, which did not accumulate in the epidermis but in the palisade mesophyll beneath. In contrast the mesophyll cells, containing chlorophyll, were generally rich in metabolites related to respiration and energy generation. As such our study emphasises the importance to study the cell-specific distribution and function of metabolites in distinguished cell types.

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