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Identification of Chlorogenic Acid as a Resistance Factor for Thrips in Chrysanthemum[C][OA]

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Western flower thrips (*Frankliniella occidentalis*) has become a key insect pest of agricultural and horticultural crops worldwide. Little is known about host plant resistance to thrips. In this study, we investigated thrips resistance in chrysanthemum (*Dendranthema grandiflora*). We identified thrips-resistant chrysanthemums applying bioassays. Subsequently, nuclear magnetic resonance (NMR)-based metabolomics was applied to compare the metabolome of thrips-resistant and -susceptible chrysanthemums. NMR facilitates wide-range coverage of the metabolome. We show that thrips-resistant and -susceptible chrysanthemums can be discriminated on basis of their metabolomic profiles. Thrips-resistant chrysanthemums contained higher amounts of the phenylpropanoids chlorogenic acid and feruloyl quinic acid. Both phenylpropanoids are known for their inhibitory effect on herbivores as well as pathogens. Thus, chlorogenic and feruloyl quinic acid are the compounds of choice to improve host plants resistance to thrips in ornamentals and crops. The effect of chlorogenic acid on thrips was further studied in bioassays with artificial diets. These experiments confirmed the negative effects on thrips. Our results prove NMR to be an important tool to identify different metabolites involved in herbivore resistance. It constitutes a significant advance in the study of plant-insect relationships, providing key information on the implementation of herbivore resistance breeding strategies in plants.

Western flower thrips (*Frankliniella occidentalis*) is a vital pest of chrysanthemum (*Dendranthema grandiflora*), an economically important ornamental for the Dutch horticultural industry (Mantel and van de Vrie, 1988). Thrips have piercing-sucking mouthparts, which enable them to feed on different types of plant cells (Hunter and Ullman, 1989). Western flower thrips can cause two types of feeding damage in chrysanthemum. Feeding on developing tissue leads to growth damage including distortion, reduction in plant growth, and eventually yields loss. Feeding on expanded tissue results in the characteristic silver damage, which affects product appearance and reduces market quality (de Jager et al., 1995a). Indirect damage is caused by transmission of tospoviruses (Maris et al., 2003).

Occurrence of host-plant resistance to thrips is sparse and little is known about the underlying mechanisms. Thrips preferentially feed on the older chrysanthemum leaves (de Jager et al., 1995a). Morphological plant characters were not involved in resistance to western flower thrips in chrysanthemum (de Jager et al., 1995a). Instead, resistance was influenced by the chemical composition of host plants, although until recently no specific compounds were identified (de Jager et al., 1995b, 1996). A novel isobutylamide was suggested to be associated with host-plant resistance to western flower thrips in chrysanthemum (Tsao et al., 2005). Overexpression of Cys protease inhibitors in transgenic chrysanthemum was not related with resistance to thrips (Annadana et al., 2002). In contrast, transgenic potato (*Solanum tuberosum*) multidomain Cys protease inhibitors were affiliated with thrips resistance (Outchkourov et al., 2004a, 2004b). Of late, two pyrrolizidine alkaloids jaconine- and jacobine-*N*-oxide and a flavanoid, kaempferol glucoside, were identified to be related to thrips resistance in hybrids of the wild plants *Senecio jacobaea* and *Senecio aquaticus* (Leiss et al., 2009).

However, as is generally the case in biological processes, it is very likely that not one but several compounds are involved in plant resistance, the identity of which are, a priori, unknown. The study of chemical host-plant resistance has so far, for technical reasons, been limited to the identification of single compounds. NMR spectroscopy allows the simultaneous detection of a wide range of metabolites, providing an instantaneous image of the metabolome of the resistant plant (Verpoorte et al., 2007). So far, NMR has been used to study the effect of pathogen infection on host plants such as phytoplasmas in *Catharanthus roseus* (Choi et al., 2004) and *Tobacco mosaic virus* in tobacco (*Nicotiana tabacum*; Choi et al., 2006). The effect

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of herbivores on plants has been studied with NMR spectroscopy for *Plutella xylostella* and *Spodoptora exigua* in *Brassica rapa* (Widarto et al., 2006) and Arabidopsis (Arany et al., 2008). We successfully applied NMR to study chemical host-plant resistance to western flower thrips in *Senecio* hybrids (Leiss et al., 2009).

In this study we investigated the metabolomic basis of thrips resistance in chrysanthemum. We first conducted an in vivo bioassay to identify thrips-resistant and -susceptible chrysanthemum cultivars, on which subsequently NMR-based metabolomics was applied. We then used an in vitro bioassay with artificial diets to confirm our findings.

RESULTS

Metabolomics

Identification of Metabolites by One- and Two-Dimensional NMR Spectroscopy

The signals of a number of metabolites present in the leaf extract of chrysanthemum, including amino acids, organic acids, carbohydrates, phenylpropanoids, and flavonoid glycosides were attributed in the ^{1}H-NMR spectra (Table I). The identification of those metabolites was based on the analysis of one- and two-dimensional NMR experiments (COSY, *J*-resolved, HSQC, HMBC), together with the comparison of reference compounds and previously reported data (Choi et al., 2006). In particular, the two-dimensional *J*-resolved experiment improved the resolution of ^{1}H-NMR spectra, allowing the attribution of the characteristic protons of phenylpropanoids in the region of phenolic signals (δ 6.0–8.5). By analysis of *J*-resolved and COSY spectra the signals from the two trans-olefinic protons of chlorogenic acid (H-7′ at δ 7.58, H-8′ at δ 6.34), 3-*O*-caffeoyl quinic acid (H-7′ at δ 7.60, H-8′ at δ 6.32), and feruloyl quinic acid (H-7′ at δ 7.62, H-8′ at δ 6.44) were clearly distinguished and attributed. In addition, two-dimensional NMR techniques allowed the assignment of the signals of a kaempferol derivative. The signals of three flavone derivatives were identified in the leaf extracts of cultivars 2 and 4 by integration of the proton signals of H-6 (δ 6.54, 6.55, and 6.56, *d*, J = 2), H-8 (δ 6.88, 6.89, and 6.90, *d*, J = 2), and H-3 (δ 6.75, 6.71, and 6.67, *s*). In the region of anomeric protons of carbohydrates, α-Glc and Suc were identified. The signal of Glc needs a special note, as the α-form of the molecule was present at very low concentration in the NMR spectra, while the β-Glc signal (δ 4.59, *d*), although the most representative in nature, was not detectable. We considered the very low amount of Glc in the extract, together with the vicinity of the residual water signal hampering the detection of β-Glc. Moreover, signals from Ala, Gln, together with those from choline, succinic acid, malic acid, and α-linolenic acid were identified in the region between 3.0 and 0.8 ppm.

Data Reduction

Metabolomics experiments produce a large number of data. Multivariate data analysis can deal with the large number of data by reducing the dimensionality of a data set. Principal component analysis (PCA) is an unsupervised method that reduces the dimensionality of a given data set by producing new linear combinations of the original variables. PCA allows the visualization of trends, clustering, similarity/dissimilarity among the samples, thus giving a macroscopic metabolic differentiation within the data set. Previous to data reduction, raw NMR data have to be bucketed to

Table I. *Characteristics of ^{1}H chemical shifts (δ) and coupling constants in metabolites of chrysanthemum leaves identified using one- and two-dimensional NMR spectra*

Metabolite	Chemical Shifts (ppm) and Coupling Constant (Hz)
Ala	1.48 (H-3, *d*, J = 7.3)
α-Linolenic acid	0.96 (H-ω, *t*, J = 7.5), 1.31 (CH_2, brs)
Gln	2.13 (H-4, *m*), 2.34 (H-3, *m*)
Choline	3.23 (*s*)
Malic acid	2.53 (H-β, *dd*, J = 5.5, 15.4), 2.78 (H-β, *dd*, J = 3.0, 15.7), 4.28 (H-α, *dd*, J = 3.0, 6.5)
Succinic acid	2.54 (*s*)
Suc	4.13 (H-1′, *d*, J = 8.5), 5.40 (H-1, *d*, J = 3.6)
α-Glc	5.24 (H-1, *d*, J = 3.7)
Fumaric acid	6.52 (*s*)
Chlorogenic acid (5-*O*-caffeoyl quinic acid)	6.34 (H-8′, *d*, J = 16.0), 6.84 (H-5′, *d*, J = 8.4), 7.03 (H-6′, *dd*, J = 8.5, 1.8), 7.13 (H-2′, *d*, J = 1.8), 7.58 (H-7′, *d*, J = 16.0)
3-*O*-caffeoyl quinic acid	6.32 (H-8′, *d*, J = 16.0), 7.60 (H-7′, *d*, J = 16.0)
5-*O*-feruloyl quinic acid	6.44 (H-8′, *d*, J = 16.0), 6.83 (H-5′, *d*, J = 8), 7.02 (H-6′, *dd*, J = 8.3, 1.5), 7.14 (H-2′, *d*, J = 1.8), 7.62 (H-7′, *d*, J = 16.0)
Kaempferol glycoside	7.12 (H-3′ and H-5′, *d*, J = 8), 8.01 (H-2′ and H-6′, *d*, J = 8)
Flavone derivative	6.54 (H-6, *d*, J = 2), 6.75 (H-3, *s*), 6.88 (H-8, *d*, J = 2)

align the signals and avoid possible fluctuations. In this study, the signals in the region between δ 0.3 and δ 10.0 were bucketed every 0.04 ppm, generating 243 variables. The generated NMR spectra were submitted to PCA analysis and thus reduced to a few principal components that explain the variation among the samples in terms of metabolic changes. A PCA model constituted by nine principal components explaining 88% of the total variance was investigated. Although not clearly separating the group of susceptible cultivars from the group of resistant lines, PCA highlighted the clustering of cultivars Oxford and Polar together, differentiating them from the rest of the samples (data not shown). According to the loading plot, the levels of chlorogenic acid, of its isomer, 3-*O*-caffeoyl quinic acid, and of feruloyl quinic acid were higher in the two resistant cultivars than in the susceptible ones and, to a minor extent, compared to the other resistant cultivars. The content of malic acid, succinic acid, Ala, and apigenin glycoside was instead higher in susceptible leaves samples (data not shown).

To distinguish resistant leaves from susceptible ones, the analysis was extended to partial least square-discrimination analysis (PLS-DA), a supervised multivariate data technique. In contrast to PCA that only uses the information of the metabolomic matrix, PLS-DA also takes into account the resistance matrix (Berrueta et al., 2007). PLS-DA uses a discrete class matrix (in this case 0 for the susceptible and 1 for the resistant group). The separation of PLS-DA is achieved by the covariance of the two datasets. When PLS-DA was applied the separation of thrips-resistant and -susceptible cultivars considerably improved (Fig. 1A). For the resistant group the validation of the PLS-DA model by permutation tests through 20 applications resulted in a variance R^2 of the model of 0.93 and a predictive ability Q^2 of the model of 0.82. For the susceptible group R^2 and Q^2 values were 0.94 and 0.79, respectively. Q^2 values greater than 0.5 are generally accepted as good (Bailey et al., 2004). All Q^2 values of the permuted Y vectors were lower than the original ones and the regression of Q^2 lines intersected

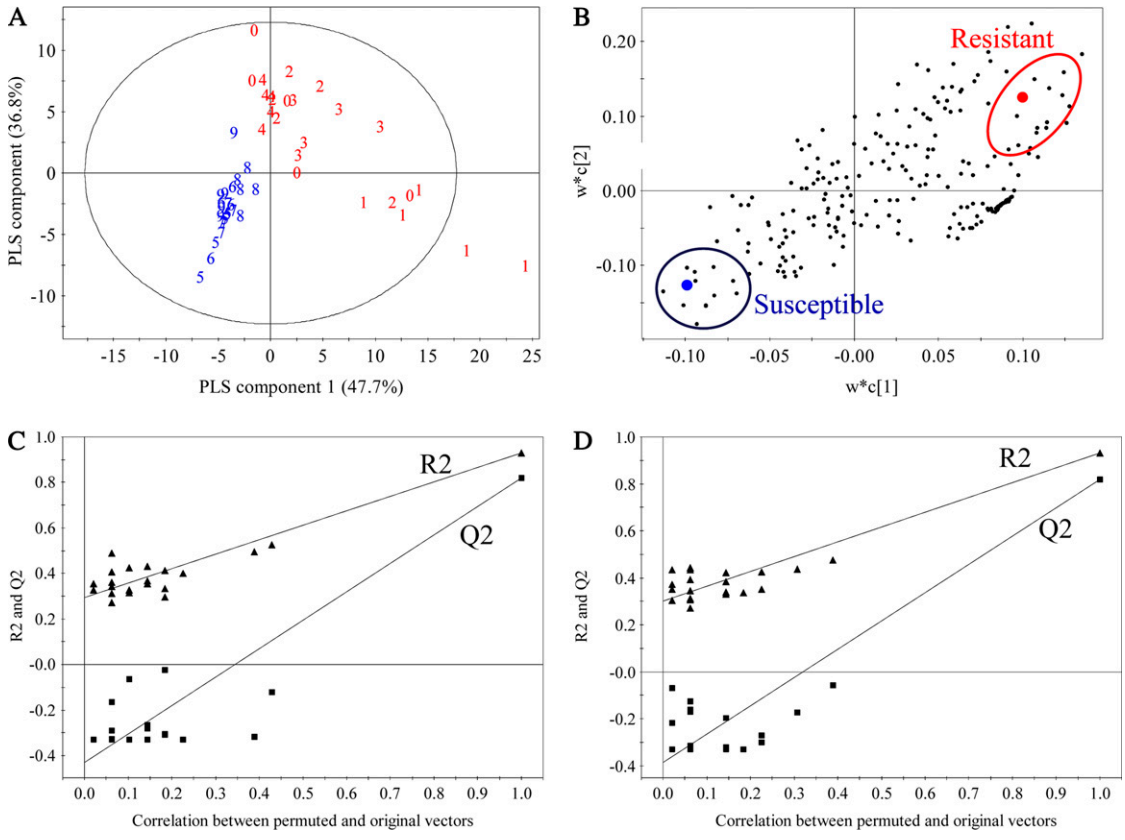

Figure 1. Score (A), loading plot (B), and permutation validation (20 permutations with three components) plot for the resistant group (C) and the susceptible group (D) of PLS-DA based on ^{1}H-NMR spectra of crude extracts from Polar (0), Oxford (1), Dublin (2), Penny Lane (3), Biarritz (4), Baykal (5), Bradford (6), Cuenca (7), Samos (8), and Super Pink Pompon (9). The PLS-DA was based on two classes of thrips-resistant (red circle) and -susceptible (blue circle) chrysanthemum lines. The ellipse in the score plot represents the Hotelling T2 with 95% confidence interval in the model (Eriksson et al., 2001). In the loading plot each dot represents the chemical shifts from bucketed ^{1}H-NMR data. The circles evidence the chemical shifts of chlorogenic acid and feruloyl quinic acid contained in the resistant samples (on the top right) and the chemical shifts of succinic acid and malic acid contained in the susceptible samples (on the bottom left). In the validation plots R^2 stands for variance and Q^2 stands for predictive ability of the model. [See online article for color version of this figure.]

at below zero (Fig. 1, C and D). Intercepts below 0.05 indicate a valid model (Eriksson et al., 2001).

According to the loading plot (Fig. 1B), chlorogenic acid and feruloyl quinic acid characterized the resistant cultivars in comparison to the susceptible ones. Susceptible cultivars showed higher contents of malic and succinic acid, together with higher NMR signals from other aliphatic compounds (in the crowded region of carbohydrates, at δ 3.0–4.5).

A *t* test was applied to the data set to determine statistically significant signals between the resistant and susceptible groups. From 243 ^{1}H-NMR signals 81 were significant ($P < 0.05$). Among them, the identified signals of chlorogenic acid, 3-*O*-caffeoyl quinic acid, feruloyl quinic acid, Ala, and malic acid, which were identified by PLS-DA, were confirmed as significantly differentiating the two groups of samples.

One of the advantages of applying NMR to metabolomics studies is that metabolites can be quantified based on their signal intensity relative to internal standard (IS; trimethylsilylpropionic acid [TMSP]). The content of the two discriminating metabolites, chlorogenic acid and feruloyl quinic acid, was thus determined by manual integration of known protons in the molecules. The signals of the two olefinic protons (H-7′ and H-8′) at 7.58 and 6.34 ppm for chlorogenic acid, and at 7.62 and 6.44 ppm for feruloyl quinic acid were considered for the calculations. Resistant chrysanthemum cultivars contained significantly more chlorogenic acid ($F = 8.46$, $df = 1$, $P = 0.017$; Fig. 2A) and feruloyl quinic acid ($F = 8.031$, $df = 1$, $P = 0.020$; Fig. 2B) compared to the susceptible cultivars. While there were no differences in the amount of these metabolites among the different susceptible cultivars, the resistant cultivars Oxford and Polar contained significantly more chlorogenic acid ($F = 14.64$, $df = 4$, $P = 0.000$) and feruloyl quinic acid ($F = 43.22$, $df = 4$, $P = 0.000$) compared to the other resistant cultivars. Both chlorogenic acid ($r = -0.780$, $n = 10$, $P = 0.008$) and feruloyl quinic acid ($r = -0.791$, $n = 10$, $P = 0.006$) were significantly negatively correlated with silver damage. They respectively explained 43% and 45% of the total variance in thrips resistance. The data for chlorogenic acid were confirmed by HPLC analysis following the method of Shao and Zhuang (2004) using the commercially available 5-*O*-caffeoyl quinic acid as standard. The extracts used for NMR analysis were thereafter used for HPLC measurement. Chlorogenic acid data measured by NMR and HPLC (data not presented) were highly conform as expressed by their significant positive correlation ($r = 0.950$, $P \leq 0.001$, $n = 10$).

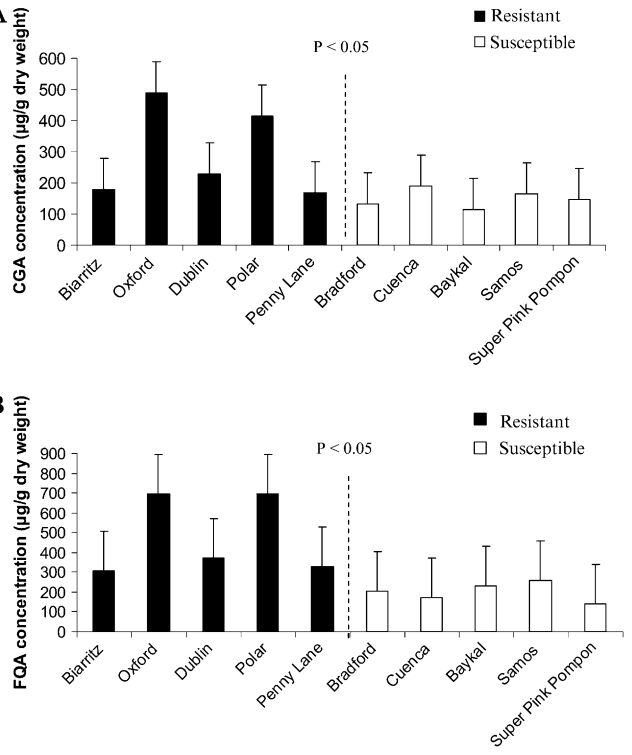

Figure 2. Quantification of chlorogenic acid (CGA; A) and feruloyl quinic acid (FQA; B) calculated on the peak intensities in ^{1}H-NMR spectra relative to an IS. Data are based on five replicates per cultivar. Means and SE of the mean are presented. Data were analyzed by ANOVA. Significant differences between resistant and susceptible cultivars are designated as $P \leq 0.05$.

Thrips Bioassays

In Vivo Thrips Bioassays

As expected, silver damage in chrysanthemum cultivars that were previously described as thrips resistant (mean of 3.56 ± 0.77) was significantly lower ($F = 15.74$, $df = 1$, $P = 0.004$) compared to cultivars that were previously described as susceptible (mean of 68.44 ± 20.18; Table II). Cultivar Polar showed the lowest silver damage (0.6 ± 0.6) within the resistant cultivars ($F = 6.28$, $df = 4$, $P = 0.002$) while cultivar Super Pink Pompon showed the highest silver damage (251 ± 41) within the susceptible cultivars ($F = 22.44$, $df = 4$, $P = 0.000$). Even when taking cultivar Super Pink Pompon out of the analysis silver damage was still significantly different between resistant and susceptible cultivars ($F = 25.19$, $df = 1$, $P = 0.002$). Growth damage did not differ between thrips-resistant (mean of 1.0 ± 0.3) and -susceptible (mean of 0.9 ± 0.2) chrysanthemum cultivars ($F = 0.003$, $df = 1$, $P = 0.955$). Number of leaves, as indicator of plant growth, was not significantly different between resistant and susceptible cultivars ($F = 0.481$, $df = 1$, $P = 0.508$). Resistant cultivars had on average 24.3 ± 0.9 and susceptible cultivars 23.0 ± 1.0 leaves, respectively.

In Vitro Thrips Bioassays

In two bioassays the relative growth rate of first instar thrips larvae was significantly reduced ($F = 5.12$, $df = 2$, $P = 0.008$ and $F = 3.43$, $df = 2$, $P = 0.037$) in the

Table II. *Silver damage (mm^2) of thrips-resistant and -susceptible chrysanthemum cultivars*

Data represent means and SEs of five replicates.

Resistant or Susceptible	Cultivar	Silver Damage
Resistant	Polar	0.6 ± 0.6
	Oxford	2 ± 0.7
	Dublin	2 ± 0.9
	Penny Lane	5 ± 2
	Biarritz	7 ± 2
Susceptible	Baykal	16 ± 3
	Bradford	23 ± 5
	Cuenca	24 ± 7
	Samos	30 ± 5
	Super Pink Pompon	251 ± 41

medium containing 5% chlorogenic acid (Fig. 3A). The mean of the relative growth rate was close to 1 (1.01 ± 0.003 and 1.04 ± 0.003), indicating no further growth. Also thrips survival was significantly reduced in both bioassays when adding 5% chlorogenic acid to the medium ($\chi^2 = 14.32$, $df = 2$, $P = 0.0008$ and $\chi^2 = 7.26$, $df = 2$, $P = 0.026$; Fig. 3B). In choice experiments first instar larvae showed a significant preference for the control over both concentrations of 1% and 5% chlorogenic (Fig. 4). This preference became significant after 1 h in the diet containing 5% chlorogenic acid and 3 h in the diet containing 1% chlorogenic acid.

DISCUSSION

In our study both the in vivo as well as the in vitro bioassays showed that chlorogenic acid is related to thrips resistance. Chlorogenic acid belongs to the naturally occurring phenolic compounds, which are known for their role in plant defense (Bennett and Wallsgrove, 1994). Plant phenolics are maintained in the nontoxic reduced state by antioxidants and stored in the cell vacuoles (Miles, 1999). These are ingested by thrips being cell feeders. When ingested by thrips the antioxidants are not renewed and phenolics become free to autooxidase or become oxidized by salivary oxidases as has been shown for aphids (Miles, 1999). Upon oxidation quinones are formed, which copolymerase with proteins (van Fleet, 1954).

Chlorogenic acid thus functions as chemical defense against herbivores due to its prooxidant effect. Chlorogenic acid is oxidized to chlorogenoquinone, which binds to free amino acids and proteins. This leads to a reduced bioavailability of amino acids and a decreased digestibility of dietary proteins (Felton et al., 1989, 1991). This negative effect on herbivores has primarily been shown for caterpillars in vitro (Bernays et al., 2000; Beninger et al., 2004) and in vivo (Elliger et al., 1981; Huang and Renwick, 1995; Mallikarjuna et al., 2004). In addition a harmful effect of chlorogenic acid has been shown for different leaf beetles (Fulcher et al., 1998; Ikonen, et al., 2002; Jassbi, 2003) as well as for a leafhopper (Dowd and Vega, 1996) and for aphids (Miles and Oertli, 1993). Chlorogenic acid not only affects the primary but also the secondary trophic level. The predator performance of a stinkbug was negatively affected by chlorogenic acid-fed prey (Traugott and Stamp, 1997). However, larvae of tobacco hornworm (*Manduca sexta*) and tobacco budworm (*Heliothis virescens*) only showed a modest growth reduction when fed on Phe ammonia-lyase modified tobacco lines (Eichenseer, et al., 1998; Johnson and Felton, 2001). These lines showed elevated levels of phenylpropanoids including a 6-fold increase in chlorogenic acid. However, high levels of oxidizable phenolics in foliage are not necessarily prooxidant (Johnson and Felton, 2001). The net oxidative balance depends on the predominant types of phenolics and phenolases present. These can vary substantially among plant species. In willow (*Salix* spp.), the response of different leaf beetles on foliar chlorogenic acid was dependant on willow species (Ikonen et al., 2001, 2002).

Miles and Oertli (1993) already suggested that a balance of reduction and oxidation, i.e. a redox system, is important in the plant defense against sucking insects. They showed that addition of the antioxidant ascorbate enhanced the negative effect of chlorogenic acid on apple aphid (*Aphis pomi*). Improvement of reducing activity by adding an antioxidant preserved

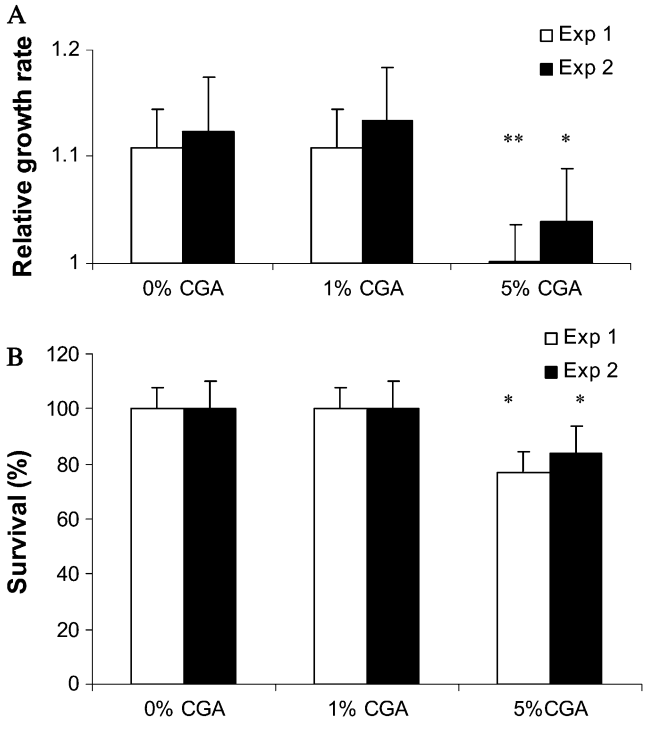

Figure 3. In vitro bioassay with first instar larvae of western flower thrips on artificial diets with 0%, 1%, and 5% chlorogenic acid (CGA). Means and SE of relative growth rate (A) and survival (B) are presented. For each diet 30 larvae were tested. The bioassay was performed twice. Asterisks indicate significant differences with ** $P \leq 0.01$ and * $P \leq 0.05$. Exp 1, Experiment 1; Exp 2, experiment 2.

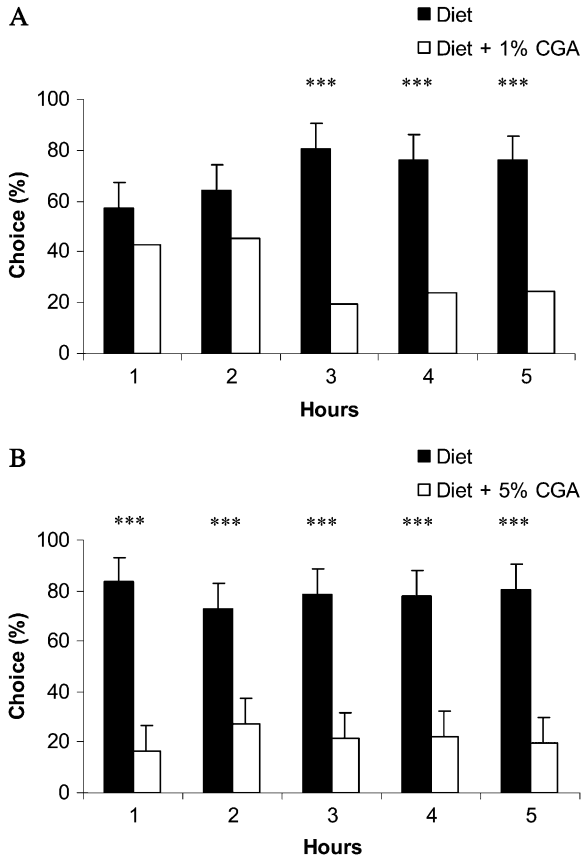

Figure 4. In vitro choice tests with first instar larvae of western flower thrips. There was a choice between an artificial diet without chlorogenic acid (CGA) and an artificial diet containing 1% (A) and 5% (B) chlorogenic acid. For each diet 30 larvae were tested. The mean and SE of the percentage larvae having made a choice for one of the diets is presented. Asterisks indicate significant differences with *** $P \leq 0.001$. Choice tests were performed twice with the same result. Here only data from the first bioassay are shown.

toxicity. Initial oxidation of chlorogenic acid to chlorogenoquinone expresses toxicity, while further oxidation considerably decreases it due to formation of phenolic oligomers (Felton and Duffey, 1991), which are much less toxic and may even act as feeding stimulants. At low concentrations the phenol catechin, a feeding deterrent in rose plants, was converted to phagostimulant polymers by salivary polyphenol oxidase of rose aphid (*Macrosiphum rosae*; Peng and Miles, 1991). This may explain why chlorogenic acid stimulated feeding of the leaf beetle *Popillia japonica* at low concentrations, while it deterred feeding at high concentrations (Fulcher et al., 1998).

Phenols may not only affect the plant dietary proteins but the proteins of the herbivores as well. Chewing insects have a peritrophic membrane that prevents many organic molecules from making immediate contact with the cells lining the midgut (Bernays, 1981). Plant-sucking insects lack such a membrane and their digestive system may thus be more vulnerable to copolymerization of proteins. However, it was suggested that salivary oxidases of aphids protect the digestive tract from copolymerization by oxidation of ingested phenolics while on their way to and within the gut (Miles, 1999).

Besides the negative effect of chlorogenic acid on herbivores it also affects fungi, bacteria, and virus such as *Phytophtora capsicii* (Lizzi et al., 1995), *Pseudomonas syringae* (Niggeweg et al., 2004), and baculovirus (Hoover et al., 1998), as well as nuclear polyhedrosis virus (Felton et al., 1986). In addition, chlorogenic acid is the most widespread natural plant dietary antioxidant (Niggeweg et al., 2004). As such chlorogenic acid is thought to prevent development of cancer and cardiovascular diseases in humans (Laranjinha et al., 1994; Sawa et al., 1999).

Another phenylpropanoid, feruloyl quinic acid, closely related to chlorogenic acid, was also identified to be involved in thrips resistance in chrysanthemum. Feruloyl quinic acid is an ester of quinic and ferulic acid. The latter is a precursor of lignin conferring rigidity to cell walls (Bennett and Wallsgrove, 1994). As such it is linked to the resistance against stem borers in maize (*Zea mays*; Santiago et al., 2006; Mao et al., 2007) and cotton (*Gossypium hirsutum*; Wang et al., 2006) as well as cereal aphids (Cabrera et al., 1995; Havlickova et al., 1996) and cereal midges (Ding et al., 2000; Abdel-Aal et al., 2001). It is also involved in the resistance to fungi such as *Fusarium gramineum* in maize (Bily et al., 2003), *Sclerotium rolfsii* in chick pea (*Cicer arietinum*; Sarma and Singh, 2003), and *Puccinia coronata* in smooth bromegrass (*Bromus inermis*; Delgado et al., 2002). Ferulic acid, being a strong antioxidant, has shown inhibiting effects on human cancer cell lines (Kampa et al., 2003; Lee, 2005).

Our approach combining in vitro and in vivo thrips bioassays with NMR was successful to show chlorogenic acid to be involved in resistance to western flower thrips in chrysanthemum. Next to chlorogenic acid, feruloyl quinic acid was implicated with thrips resistance. Both metabolites are not only implicated with the resistance to insects but also to fungi and in the case of chlorogenic acid to bacteria and virus. This may form the basis of a multiresistance breeding program. In addition, due to the unique combination of negative effects on thrips with positive effects on human health chlorogenic and feruloyl quinic acid are the compounds of choice to improve host plant resistance. Indeed, additive genetic inheritance of chlorogenic acid has been shown (Ky et al., 1999). Besides, Niggeweg et al. (2004), for dietary purposes, engineered tomatoes (*Solanum lycopersicum*) with a doubled amount of chlorogenic acid, without influencing other phenylpropanoids, by overexpression of hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase. Our results prove NMR a promising tool to identify different metabolites involved in herbivore resistance. It constitutes a significant advance in the study of plant-insect relationships, providing key information on the implementation of herbivore resistance breeding strategies in plants.

MATERIALS AND METHODS

Plant Materials

Commercially produced cuttings of five chrysanthemum cultivars resistant for silver damage (cultivars Biarritz, Oxford, Dublin, Polar, and Penny Lane) and five cultivars susceptible to silver damage (cultivars Bradford, Cuenca, Baykal, Samos, and Super Pink Pompon) derived from the Dutch chrysanthemum breeder Deliflor were used for this study. Plants were described as thrips resistant or susceptible based on an earlier choice bioassay we conducted for Deliflor on a commercial basis. Plants were potted into 11 cm diameter pots filled with equal parts of dune sand and potting soil in spring 2008. Ten replicates of each cultivar were transferred to a growth chamber (L:D, 18:6, 20°C:15°C) and grown for 6 weeks. Five replicates were used for the thrips bioassay while the other five replicates were used for the NMR metabolomics.

In Vivo Thrips Bioassay

Five 6-week-old vegetative plants of each chrysanthemum cultivar were tested in a nonchoice bioassay. Each plant was placed into individual thrips-proof cages, consisting of plastic cylinders (80 cm height, 20 cm diameter), closed on both ends with displaceable rings of thrips-proof gaze. The cages were arranged in a fully randomized design and to each cage 10 adult western flower thrips were added and left for 1 week. Thereafter, silver damage, expressed as the leaf area damaged in mm^2, and growth damage, expressed as the number of leaves with distortions, was visually scored for each plant. The number of leaves for each plant was counted. Silver damage did not follow a normal distribution and was, therefore, ln transformed. Data were analyzed using a nested ANOVA (Sokal and Rohlf, 1995) with cultivars nested within susceptible and resistant chrysanthemums, respectively.

Metabolomics

Extraction of Plant Material

Five plants each of the five resistant and the five susceptible chrysanthemum cultivars were used for NMR metabolomics. Plants were grown under standard conditions, described above, for 6 weeks. From each individual plant the third leaf from below was taken for analysis. Thus five replicate leaves per cultivar were used. The plant material was immediately frozen in liquid nitrogen upon harvesting and stored at −80°C until extraction. Each sample was ground under liquid N_2 and freeze dried. Freeze-dried plant material (50 mg) was transferred to a 2-mL microtube. A volume of 1.5 mL of a mixture of KH_2PO_4 buffer (pH 6.0) in D_2O containing 0.05% trimethyl silyl propionic acid sodium salt (w/v; TMSP) and methanol-d_4 (1:1) was added to the plant samples. The mixture was vortexed at room temperature for 1 min, ultrasonicated for 20 min, and centrifuged at 13,000 rpm for 15 min. An aliquot of 0.8 mL was used for NMR analysis.

NMR Analysis

NMR spectra were recorded at 25°C on a 600 MHz Bruker DMX-600 spectrometer (Bruker) operating at a proton NMR frequency of 600.13 MHz. MeOH-d_4 was used as the internal lock. Each ^{1}H-NMR spectrum consisted of 256 scans requiring 8 min and 30 s acquisition time with the following parameters: 0.12 Hz/point, pulse width of 30 (11.3 μs), and relaxation delay of 1.5 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 decay was Fourier transformed with a line broadening factor of 0.3 Hz. The resulting spectra were manually phased and baseline corrected, and calibrated to the IS trimethyl silyl propionic acid sodium salt (TMSP) at 0.0 ppm using TOPSPIN (version 2.0, Bruker). Two dimensional *J*-resolved NMR spectra were acquired using 32 scans per 64 increments for F_1 and 1,638.4 k for F_2 using spectral widths of 6,009.6 Hz in F_2 (chemical shift axis) and 50 Hz in F_1 (spin-spin coupling constant axis). A 1.5 s relaxation delay was employed. Datasets were zero filled to 512 points in F_1 and both dimensions were multiplied by sine-bell functions (spinning sideband = 0) prior to double complex Fourier transform. *J*-resolved spectra were tilted by 45°, symmetrized about F_1, and then calibrated to TMSP, using TOPSPIN (version 2.0, Bruker). The COSY spectra were acquired with a 1.0 s relaxation delay and 6,009.6 Hz spectral width in both dimensions. The window function for the COSY spectra was Qsine (spinning sideband = 2.0). The HSQC spectra were obtained with a 1.0 s relaxation delay and 6,009.15 Hz spectral width in F_2 and 164 Hz in F_1. The HMBC spectra were recorded with a 1.0 s relaxation delay and 31,692.7 Hz spectral width in F_2 and 164 Hz in F_1. The optimized coupling constants for HSQC and HMBC were 140 and 10 Hz, respectively.

Quantification

For the quantification of chrysanthemum metabolites using NMR spectroscopy, the peak area of selected proton signals belonging to the target compounds, and the peak area of IS, TMSP, were integrated manually for all the samples. The following equation was applied for the calculations (adjusted from van Beek et al. [1993]):

$$c = \frac{\text{integral(Target)}}{\text{integral (IS)}} \times \frac{^{*}\text{MW(Target)}}{^{**}\text{MW (IS)}} \times \text{weight (IS)}$$

MW = M_r: *, divided by the number of protons involved in the target signal (Target); **, divided by the number of protons involved in the IS signal (IS). c = concentration (μg/50 mg). Weight is in micrograms.

The concentration of TMSP in each NMR tube was fixed as 1.55 μmol, from which the weight of IS (in μg) was calculated.

Data Analysis

Spectral intensities of ^{1}H-NMR spectra were scaled to the intensity of the IS (TMSP, 0.05% [w/v]) and reduced to integrated regions of equal width (0.04) corresponding to the region of δ 0.4 to δ 10.0. The regions of δ 4.8 to δ 4.9 and δ 3.28 to δ 3.40 were excluded from the analysis because of the residual signal of water and MeOH. PCA and PLS-DA were performed with the SIMCA-P software (v. 11.0, Umetrics). The scaling method for PCA was Pareto and for PLS-DA the unit-variance method. The *t* test was performed by Multi Experiment Viewer (v. 4; Saeed et al., 2003). The PLS-DA model was validated using the permutation method through 20 applications, which is a default validation tool in the software package applied (SIMCA-P). Variance (R^2) and cross-validated variance values (predictive ability of the model, Q^2) of PLS-DA using five components were calculated.

In Vitro Thrips Bioassays

Special observation plates of clear plastic, as described by de Jager et al. (1995b) were used to study larval performance on liquid media with different chlorogenic acid concentrations. A general insect diet developed by Singh (1983) was modified, taking only the soluble ingredients, and used as liquid medium. The liquid medium was placed in small cups of a bottom plate and covered with stretched parafilm, through which thrips are able to feed (de Jager et al., 1995b). Bioassays and choice tests were performed. For the bioassays a middle plate with six wholes was placed on top of the parafilm to keep the thrips larvae separated. The middle plate was covered by a top plate. Bioassays were conducted with concentrations of 0%, 1%, and 5% chlorogenic acid added to the liquid medium. Thirty first instar larvae of western flower thrips were introduced on each medium and their length was measured. After 3 d, at the end of the first larval instar period, their length was remeasured and their relative growth rate calculated. Also the percentage of surviving larvae after 3 d was determined. For the choice tests one-half of the plates was filled with artificial diet without chlorogenic acid while the other half contained liquid medium to which chlorogenic acid in concentrations of 1% or 5% had been added. A middle plate with three arenas comprising one cup each of the different media was placed on top of the parafilm and covered by a glass plate. Sixty first instar larvae were introduced. The choice of the thrips larvae was recorded every hour up to 5 h after introduction of larvae. The in vitro tests were performed in a growth chamber under standard rearing conditions (L:D 12:12, 23°C:23°C). Each bioassay and each choice test was performed twice. A one-way ANOVA was performed to analyze relative growth rates in the bioassays, while survival was analyzed by a chi-square test. In the choice tests data were analyzed for each hour using a binomial test (Sokal and Rohlf, 1995).

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