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An analysis of plant–aphid interactions by different microarray hybridization strategies

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Abstract

Aphids have long been considered 'stealthy' herbivores that subvert a plant's induced defenses and manipulate its source-sink signaling, but these hypotheses are largely untested at a transcriptional level. We analysed gene expression in native tobacco plants (*Nicotiana attenuata*) infested with *Myzus nicotianae* aphids, without resorting to the use of clip-cages, with a cDNA microarray containing 240 defense-related *N. attenuata* genes. Using a hybridization scheme ('ratio analysis' and 'state analysis') broadly applicable in two-factor analyses, we examined how the aphids influenced source–sink relationships and determined if their feeding preference, apart from benefiting from the sink strength of young leaves, was associated with the expression of known plant defense genes. In contrast to the responses elicited by attack from tissue-feeding lepidopteran larvae and mesophyll-sucking insects, attack from phloem-feeding aphids elicited only weak responses. Similar to other herbivores, *M. nicotianae* feeding increased the expression of trypsin protease inhibitors (TPI), lipoxygenase, and xyloglucan-endotransglycosylase genes, and decreased small RUBISCO subunit and ubiquitin carrier protein transcripts. Aphid-specific changes included the up-regulation of glutamate synthase and the down-regulation of a germin-like protein. Aphids preferentially settled on younger leaves, which expressed more hydroperoxide lyase and TPI than did older leaves, suggesting that these genes, which mediate the synthesis of compounds reported to be toxic for aphids in other plant systems, are either not under transcriptional control or not important in this system. By identifying aphid-responsive genes, we have made a first step in identifying the 'genes that matter' in plant–aphid interactions.

Keywords: feeding guild, feeding preference, *Myzus persicae*, source-sink manipulation

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Introduction

From a plant's perspective, phloem feeders, such as aphids, or phloem-parasitizing plants (e.g. *Cuscuta* spp., *Orobanche* spp.) are sinks for sugars and nutrients similar to newly expanding leaves, developing buds or maturing fruits. However, phloem sap is the 'junk food' of plant diets, rich in carbohydrates but very low in protein and amino acids (aa) (Sandstrom & Moran 1999). To cope with this unbalanced diet, aphids consume large amounts of phloem sap, excrete the excess carbohydrates as 'honeydew', scavenge the nitrogen-containing constituents, and house bacterial endosymbionts that provide the aphids with essential aa. Some aphid species manipulate aa composition in the phloem (Sandstrom *et al*. 2000); others, such as gall-forming *Pemphigus betae* aphids, manipulate plant allocation patterns while competing with plant sinks for resources (Larson & Whitham 1997, and references therein). The mechanisms responsible for these manipulations are largely unknown. Microarrays provide the opportunity to monitor transcriptional responses of a large number of genes and are increasingly being used to study plant–insect interactions (Arimura *et al*. 2000; Reymond *et al*. 2000; Hui *et al*. 2003). Using a cDNA array containing genes from a native tobacco that are differentially regulated by attack from leaf-chewing and single cell-feeding herbivores (Voelckel & Baldwin 2004), we determine whether or not aphids manipulate transcriptional processes in sink and source tissues of their host plant.

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Studies that have examined aphid-induced alterations in plant gene expression include Fidantsef *et al*. (1999) who compared the effects of phloem feeders to chewing insects on tomato plants. They found that after *Macrosiphium euphorbiae*/*Myzus persicae* attack, lipoxygenase (LOX) and pathogenesis-related protein P4 (PR1) were strongly elicited but proteinase inhibitor (PI) II was not expressed, whereas after *Helicoverpa zea* attack, the opposite occurred. Similarly, the transcriptional signatures of salicylic acid signaling (apoplastic β-1,3-glucanase, PR-1) and to a lesser degree the signatures of jasmonic acid /ethylene-signaling (antimicrobial defensin PDF1.2) and wound signaling (LOX2, but not LOX1) were found in *M. persicae*-attacked *Arabidopsis thaliana* plants (Moran & Thompson 2001). *Myzus persicae*-mediated increases in phenylalanine ammonia lyase (PAL1) and monosaccharide symporter mRNAs suggest imbalances of phenolics and sugars at the wound site as a result of sequestration of phenolics in stylet sheaths and the generation of metabolic sinks, respectively (Moran & Thompson 2001).

Examining the *M. persicae–Arabidopsis* interaction further using arrays, Moran *et al*. (2002) discovered many diverse responses. For example, oxidative stress genes (glutathione-*S*-transferases, superoxide dismutases), Ca^{2+}/calmodulin-related signalling genes, PR genes (BGL2, PR-1, hevein-like protein), ethylene biosynthesis genes (ACC oxidase 1), aromatic biosynthesis genes (PAL2, chalcone synthase, tyrosine decarboxylase), and tryptophan biosynthetic pathway genes (anthranilate synthase β-subunit, tryptophan synthase) were found to be up-regulated or down-regulated after 72–96 h of *M. persicae*-attack. A similar comprehensive array-analysis was used by Zhu-Salzman *et al*. (2004) to compare the transcriptional responses in *Sorghum bicolor* plants elicited either by greenbugs (*Schizaphis graminae*), salicylic acid (SA) or jasmonic acid (JA). Greenbug attack caused changes in the expression of defense genes (PRs, PIs, phenolics biosynthesis genes), antioxidant genes (glutathione-*S*-transferases, lactoylglutathione lyase, catalase), abiotic stress-related genes (drought-, salt- and low-temperature-responsive genes, aldehyde oxidase), nitrogen-assimilation genes (nitrite reductase), photosynthesis genes and genes of unknown function (two of which were greenbug-specific). While some PR genes (thaumatin like proteins) responded more strongly and more rapidly to greenbug attack than to salicylic acid, jasmonic acid-regulated genes (LOX, a cytochrome P450, dhurrinase, PI) were only marginally and transiently induced by the aphid, as revealed by Northern blot analysis. The latter also identified two additional greenbug-specific genes; a leucine-rich repeat-containing protein and a defense-related protein (DRP) known to be induced by sugar depletion. Zhu-Salzman *et al*. (2004) interpret the induction of DRP in the light of a fourfold decrease in soluble carbohydrate concentration in greenbug-infested barley (Cabrera *et al*. 1994). Finally, a faster and stronger accumulation of PR-1 transcripts was found in incompatible compared with compatible *M. euphorbiae–Lycopersicon esculentum* interactions (de Ilarduya *et al*. 2003).

In summary, phloem feeders often elicit the transcriptional signature of SA- and pathogen signaling. This elicitation may reflect responses to virus vectoring by aphids and whiteflies or aphid-associated bacterial endosymbionts, or it may suggest the similarities of intercellular fungal hyphae growth and aphid stylet penetration (Fidantsef *et al*. 1999; Walling 2000; McKenzie *et al*. 2002). The limited elicitation of JA-mediated defense responses may be due to antagonistic crosstalk with SA and ethylene signaling or stealthy feeding behavior which minimizes the amount of tissue damaged (Zhu-Salzman *et al*. 2004).

Plant–aphid interaction studies frequently used Tanglefoot or clip cages to confine insects to leaves. Clip cages are known to decrease CO_2-exchange rates and soluble leaf protein in cotton (Crafts-Brandner & Chu 1999), indicating their adverse effects on plant metabolism. Here, we take advantage of the strong feeding preference of *Myzus nicotianae* for young leaves in order to examine local and systemic plant responses without the potential confounding influence of clip-cages. We use a native tobacco (*Nicotiana attenuata*) and analyse its responses to a naturally occurring tobacco aphid (*M. nicotianae*) with a cDNA microarray enriched in defense-related genes. We ask the following questions: (1) Does *M. nicotianae*, a phloem feeder, elicit transcriptional changes that are different from those elicited by representatives of other feeding guilds, such as *Manduca sexta*, a leaf chewer, and *Tupiocoris notatus*, a cell-content feeder (Voelckel & Baldwin 2004), and if so, what is the nature of these specific changes? (2) Can we detect differences in defense gene expression between sources and sinks that explain feeding preferences for sink leaves? (3) Can we detect transcriptional evidence that aphids manipulate source–sink relationships?

Array-analyses based on competitive hybridizations of two differentially labeled cDNAs allow for different sets of binary comparisons. How a factor, e.g. aphid herbivory, modulates gene expression in the absence of this factor is a question readily answered using one array (referred to as 'state analysis'). How a relative gene expression ratio, e.g. gene expression in sink relative to source leaves, is modulated by insect herbivory is a second a question, which requires the use of two arrays (referred to as 'ratio analysis'). We use both approaches and consider their respective advantages and disadvantages.

Materials and methods

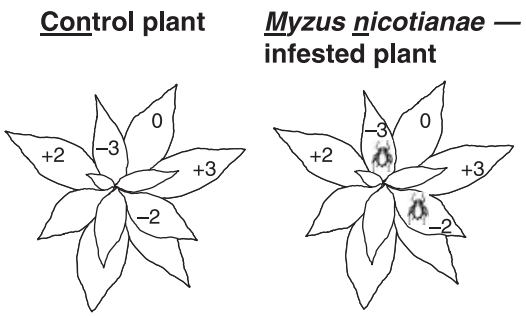
Plant and insect cultivation

Seeds of an inbred line of *N. attenuata* Torr. Ex Wats. (synonymous with *Nicotiana torreyana* Nelson and Macbr.),

which was originally collected in south-western Utah, USA, in 1992, were germinated and grown hydroponically as described by Hermsmeier *et al*. (2001). Throughout the experiment plants were grown at 26–28 °C under 16 h of light. A day before placing aphids on plants, 1 mL of 1 M KNO_3 was added to each 1-L hydroponic chamber, and 36 randomly chosen rosette-stage plants were paired by rosette size.

A red strain of *M. nicotianae* aphids, initially obtained from a *N. tabacum* field near Heidelberg, Germany, was bred for several generations on glasshouse-grown *N. attenuata* plants before being used in this experiment. Formerly considered a tobacco-adapted form of generalist *M. persicae*, tobacco-associated aphids were described as *M. nicotianae* in 1987 (Blackman 1987). However, recent genetic, biochemical, and behavioral evidence suggests that *M. nicotianae* and *M. persicae* are conspecifics (Clements *et al*. 2000a,b). In the glasshouse, *M. nicotianae* has been observed to preferentially aggregate on bolting tissue and younger leaves, as reported in the literature (*M. persicae*, Moran & Thompson 2001). In a preliminary experiment, we monitored aphid movements within rosette-stage plants over 2 d. Apterous adult females placed on each of two sink leaves (the second and the third younger than the source–sink transition leaf, designated as leaves at nodes –2 and –3, respectively) were observed to remain and reproduce on these leaves or move toward the center of the rosette to newly expanding leaves (data not shown). We used this feeding preference for young leaves to avoid the use of clip cages. One plant in each pair of experimental plants was infested with two apterous females on each of the leaves at nodes –2 and –3 (infested plant), while the other plant received no aphids (control plant). The location of each of the four aphids per infested plant was monitored twice daily; if an aphid had moved to younger leaves or had died, it was replaced with a new one to ensure a constant aphid density on the local leaves throughout the 48-h experiment. Plants typically supported a population of four to eight females plus nymphs on their sink leaves. After 2 d of feeding, all aphids were removed and the two attacked leaves (–2 and –3) and two unattacked, source leaves (+2 and +3) were harvested from all plants and pooled separately from infested and uninfested plants. Hence, four samples (local infested, systemic infested, local control, systemic control) from 18 replicate plants were obtained (Fig. 1). Leaves were flash-frozen in liquid nitrogen and stored at –80 °C until used in microarray analysis.

(a)



–2, –3	local leaves (sinks)
+2, +3	systemic leaves (sources)
0	source–sink-transition leaf

(b)

slide	Cy5		Cy3	
1	con	loc	*M. n.*	loc
2	con	sys	*M. n.*	sys
3	con	sys	con	loc
4	*M. n.*	sys	*M. n.*	loc

Fig. 1 (a) Experimental design: two sink leaves (–2, –3; local) of 18 plants were infested each with two female, viviparous adult *Myzus nicotianae* and harvested after 48 h as well as two uninfested, source leaves (+2, +3; systemic) from the same plants. Corresponding sink and source leaves from 18 control plants were harvested in parallel. (b) Scheme of hybridizations depicting the sources of Cy5- and Cy3-labelled cDNA of the four binary comparisons. With slides 1 and 2, local (loc) and systemic (sys) leaves of infested plants are directly compared with their counterparts on uninfested (con) plants ('state analysis'). On the remaining slides the relative gene expression between sink and source leaves is evaluated ('ratio analysis'): without aphids (slide 3) and with aphids (slide 4).

cDNA-array fabrication, hybridization, and quantification

A total of 234 genes that were cloned by differential display reverse transcription — polymerase chain reaction (DDRT-PCR), subtractive hybridization with magnetic beads (SHMB), and cDNA-amplified fragment length polymorphism (AFLP) from *N. attenuata* plants subjected to real and simulated herbivory from *M. sexta* larvae (Hermsmeier *et al.* 2001; Halitschke *et al.* 2003; Hui *et al.* 2003) and six well-characterized *Manduca*-induced genes (*PMT*, *XTH*, *AOS*, *HPL*, *TPI* and *WRKY*) were PCR-amplified and spotted on epoxy coated slides as described in Halitschke *et al*. (2003). For each cDNA, two PCR fragments, with 5'-aminolink on either strand, were synthesized, and each PCR fragment was spotted four times. Hence, each gene was represented by two independent PCR fragments, which, in turn, were spotted in quadruplicate. A complete list of positions and identities of PCR products spotted on the cDNA-array can be found in supplementary materials (SupplMat1, 2).

Samples were ground under liquid nitrogen and total RNA was isolated according to the methods of Pawlowski *et al*. (1994). Four hybridizations were performed and cDNAs were labeled with either Cy3 or Cy5 fluorescent

dyes as specified in Fig. 1b. For cDNA synthesis, Cy3/Cy5 labeling, hybridization procedures, array scanning, evaluation of images and signal strengths (AIDA Image Analyser and AIDA software package; Raytest Isotopenmessgeräte GmbH, Straubenhardt, Germany), and calculation of array-specific normalization factors and normalized Cy3/Cy5 ratios, see Halitschke *et al*. (2003).

Criteria for differential expression

Normalized Cy3/Cy5 ratios for each individual spot (expression ratio: ER) and the mean of the four replicate spots for each cDNA (two for each gene: mean ER1, mean ER2) were calculated. Mean ERs that included negative values as well as obvious outliers were excluded from further analysis. A transcript was defined as being differentially regulated if both of the following criteria were fulfilled: (1) the final ER ((mean ER1 + mean ER2)/2) was equal to or exceeded the arbitrary thresholds (≤ 0.77 for down-regulated genes ($\log 0.77 = -0.11$) or ≥ 1.3 for up-regulated genes ($\log 1.3 = 0.11$); (2) mean ER1 and mean ER2 were significantly different from 1 as evaluated by *t*-tests to control for ER-variance and ER-sample size. For justification and evaluation of these criteria see Halitschke *et al*. (2003). Original data are organized according to their order in Figs 2 and 3 (see SupplMat3).

Results

In a previous study, the cDNA array was competitively hybridized with RNA from uninfested plants and RNA stemming from whole-plant infestations with either *M. sexta* (leaf chewer) or *T. notatus* (cell content feeder) (Voelckel & Baldwin 2004). *Myzus nicotianae* aphids elicited substantially fewer genes than did attack from these two species and the plants' responses to these two herbivores were more similar than either response was to the response to aphids (Fig. 2). Aphid attack did not elicit (mentioning only the prominent differences): a burst in threonine deaminase (TD) mRNA levels, an increase in allene oxide synthase, α-dioxygenase (α-DOX), hydroperoxide lyase (HPL), a pto-responsive gene, or a light-harvesting complex gene. Neither did it decrease the expression of glycine hydroxymethyltransferase, histone 3, heatshock protein 70, a GTP-binding protein, a metallothionein, a Gap dehydrogenase, or a protein translation factor (SupplMat4). Considering the differences in sampling time (24 h in the *M. sexta*/*T. notatus* experiment and 48 h in the *M. nicotianae* experiment), sampled tissue (whole rosette vs. two specific leaf positions), and herbivore infestation densities (15–20 vs. 4–8 individuals), this comparison may overestimate or underestimate differences in gene expression.

Since *M. nicotianae* preferentially attacks young leaves, constitutive defense gene expression in younger relative to older leaves may correlate with this feeding preference. Moreover, constitutive expression patterns may be actively manipulated by aphids. To test these hypotheses, two hybridizations were performed as described in Fig. 1 (slides 3 and 4), and the results of this 'ratio analysis' are summarized in Fig. 3.

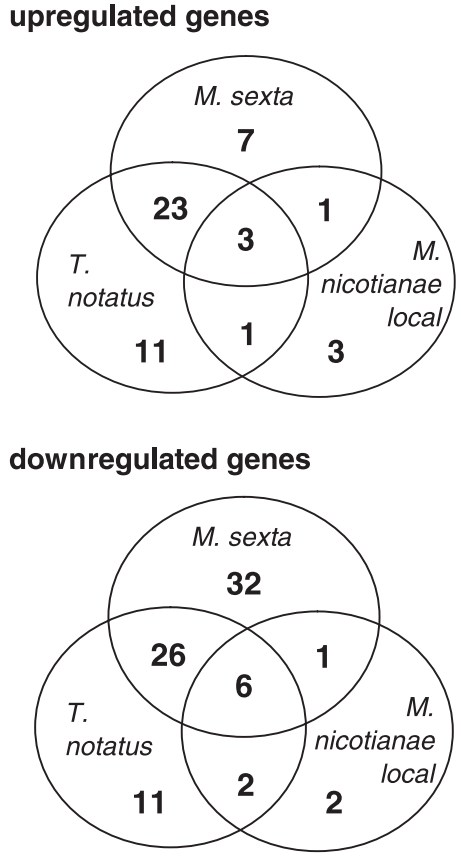
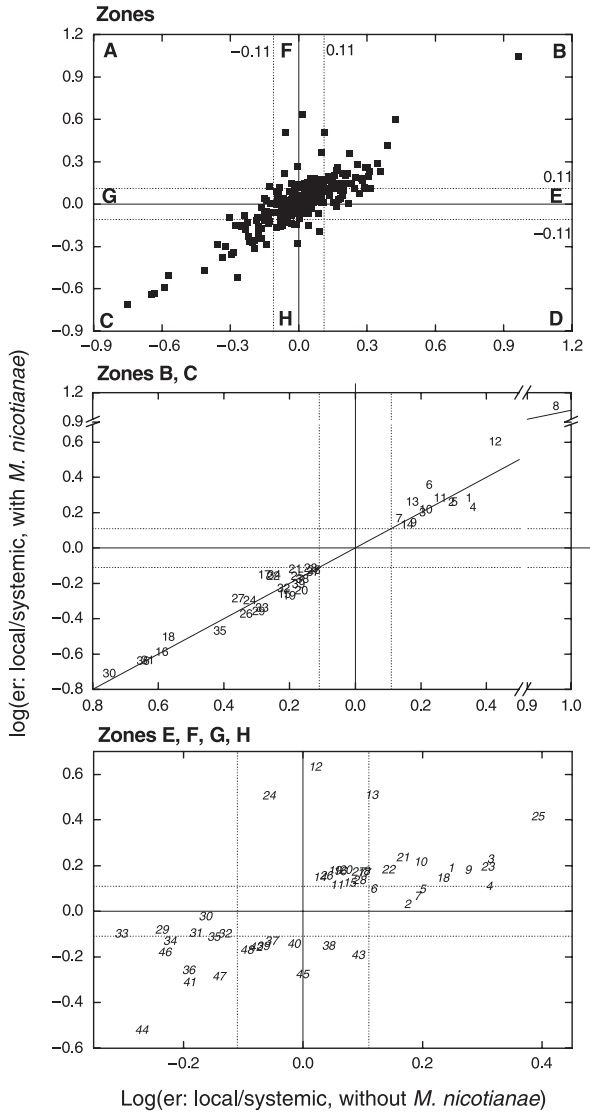


Fig. 2 Venn diagram depicting the number of cDNAs showing common or differential expression in response to attack by a leaf chewer (*Manduca sexta*), a cell-content feeder (*Tupiocoris notatus*), and a phloem-feeder (*Myzus nicotianae*). For details see Supplementary Material (SupplMat4).

Interestingly, in control plants, the expression of defense genes such as *TPI* and *HPL*, photosynthesis genes such as chelatase, RUBISCO ssu, O_2-evolving and light-harvesting complex proteins, and 12 unknown genes was higher in sink leaves than it was in source leaves. In contrast, source leaves expressed defense-related genes, such as α-DOX, 13-lipoxygenase (LOX), TD, epi-aristolochene synthase, luminal binding protein (BiP), and other genes such as a senescence-up-regulated protein, a protein translation factor, an ubiquitin carrier protein, histone 3, a MAR-binding protein and 14 unknown genes to a greater extent than did sink leaves.

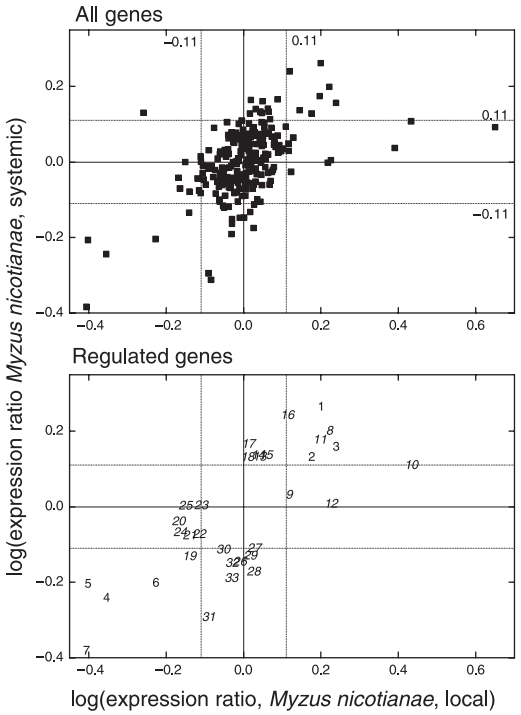
While these sink–source expression differences remained in most cases (Fig. 3, upper half of table), in some cases (Fig. 3, lower half of table), the differences vanished in response to aphid attack (e.g. light-harvesting- and



mRNA levels in sink- compared to source-leaves			
Description	Label	– *M.n.*	+ *M.n.*
Genes not influenced by *M. nicotianae* attack (Zones B+C)			
18S rRNA	2	high	high
hydroperoxide lyase (HPL)	9	high	high
proteinase inhibitor (PI)	8	high	high
Mg protoporphyrin IX chelatase	4	high	high
pore protein	14	high	high
ssu pseudogene	11	high	high
9 unknowns	1, 3, 5-7, 10, 12, 13	high	high
ubiquitin carrier protein	24, 25	low	low
alpha-dioxygenase	16, 36	low	low
metallothionein-like protein	30, 31	low	low
5-epi-aristolochene synthase	28	low	low
C-7	32, 34	low	low
PII10	26	low	low
luminal binding protein (BiP)	17	low	low
putative protein translation factor SUI	38	low	low
senescence upregulated protein	18	low	low
12 unknowns	15, 19, 20-23, 27, 29, 33, 35, 37, 39	low	low
Genes influenced by *M. nicotianae* attack (Zones E, F, G, H)			
beta-tubulin	1	high	equal
Ihcb1	2	high	equal
PSII O2 evolving complex peptide	4	high	equal
chloroplast genome DNA	6	high	equal
3 unknowns	3, 5, 7	high	equal
60S ribosomal protein	23	equal	high
Sn-1 gene	8	equal	high
xyloglucan endo-transglycosylase B1	12, 13, 24	equal	high
ripening regulated protein	26	equal	high
26S rRNA	11	equal	high
Fd-GOGAT precursor	9	equal	high
actin gene Sfa 15B	19	equal	high
12 unknowns	10, 14-18, 20-22, 25, 27, 28	equal	high
13-lipoxygenase	33	low	equal
threonine deaminase	29	low	equal
MAR-binding protein	32	low	equal
histone H3	31	low	equal
2 unknowns	30, 34	low	equal
germin homolog	37	equal	low
ITS 26S + 18S	43	equal	low
WRKY2	46	equal	low
HMGR	44	equal	low
major intrinsic protein 2	45	equal	low
9 unknowns	35, 36, 38-42, 47, 48	equal	low

Fig. 3 Left panel: analysis of the relative expression of defense-related transcripts in sink compared with source leaves (*x*-axis) and the influence of *Myzus nicotianae*, a phloem feeder, on this relative expression (*y*-axis). The upper graph depicts the mean ratio of all 240 genes and distinguishes individual zones of which the relevant ones are depicted separately in the graphs below. The lower graphs show only differentially expressed genes (i.e. genes fulfilling the criteria specified in the Materials and Methods). Right panel: identification of genes from the graphs. Independent of *M. nicotianae* attack, a number of genes are constitutively more highly expressed in sink leaves than in source leaves (Zone B) and vice versa (Zone C). For some genes this differential expression between sinks and sources is removed when *M. nicotianae* attacks plants (Zones E and G); for others with no initial difference in constitutive sink- and source-expression, *M. nicotianae*-attack elicits differential expression (Zones F and H).

O_2-evolving complex proteins, LOX and TD). Moreover, some genes that had displayed no differences in expression between sink and source leaves before aphid attack (ratio = 1) showed higher expression in sink [ratio > 1, e.g. xyloglucan-endotransglycosylase (XTH) and glutamate synthase (Fd-GOGAT)] or source (ratio < 1, e.g. germin and HMGR) leaves after aphid attack (Fig. 3, lower half of table). Regardless whether aphid attack erases the normal sink–source difference in expression of a gene (ratio ≠ 1 → ratio = 1, e.g. TD) or elicits a difference in sink/source expression when none existed (ratio = 1 → ratio ≠ 1, e.g. germin), this analysis can not determine whether the difference results from a change in sink or in source leaves. For example, in case of LOX (< 1 → 1), we cannot discern whether aphid attack caused an increase in sink expression or a decrease in source expression. Other ratio changes (1 → < 1, 1 → > 1, and > 1 → 1) remain similarly irresolvable. Because of these limitations inherent to 'ratio' analysis, we performed another set of comparisons, referred to as 'state' analysis (Fig. 4).



Description	Label	local	systemic
locally and systemically regulated			
18S rRNA	2	up	up
trypsin inhibitor (TPI)	3	up	up
unknown	1	up	up
ITS 26S-18S	6	down	down
small subunit of rubisco	4	down	down
ssu pseudogene	5, 7	down	down
locally or systemically regulated			
13-lipoxygenase	12	up	non
Fd-GOGAT	8	up	non
Mg protoporphyrin IX chelatase	9	up	non
xyloglucan endo-transglycosylase B1	10	up	non
unknown	11	up	non
triosephosphate isomerase	18	non	up
5 unknowns	13-17	non	up
germin	19	down	non
lhbC1	20	down	non
putative 60S ribosomal protein	23	down	non
ssu pseudogene	25	down	non
ubiquitin carrier protein	22	down	non
unknown	21, 24	down	non
alpha-dioxygenase	28	non	down
HMGR	32	non	down
metallothionein-like protein	33	non	down
PII10	31	non	down
senescence upregulated protein	29	non	down
3 unknowns	26, 27, 30	non	down

Fig. 4 Left panel: logarithms of mean gene expression ratios (ERs) derived from the array-analysis of locally *Myzus nicotianae* infested (*x*-axis) vs. systemically uninfested leaves (*y*-axis) of the same plant. The upper graph depicts the mean ratios of all 240 genes. In the lower graph only those genes are depicted whose logarithmic mean ERs exceed the arbitrary thresholds (log 1.3 = 0.11, log 0.76 = −0.11) and differ significantly from 1, as evaluated by *t*-tests. Roman numbers indicate the fulfilment of these criteria for local and systemic expression; italic numbers indicate the fulfilment of these criteria for either local or systemic expression. Right panel: Identification of genes from the graphs and their regulation pattern (local, systemic, up, down, non).

LOX, Fd-GOGAT, and XTH were locally up-regulated whereas germin and light-harvesting complex protein were locally downregulated (Fig. 4, lower half of table). These results not only explain which type of regulation led to the change in the sink–source expression ratio as revealed by the previous 'ratio analysis', but also serve as an indirect replication of the analysis. Not all patterns of expression, however, were as neatly replicated (Fig. 4, lower half of table): for example, the systemic up-regulation of triose phosphate isomerase ('state analysis') did not lead to a change in its sink–source ratio ('ratio analysis'). The behavior of two other genes (local down-regulation of a ribosomal protein, systemic down-regulation of HMGR) even contradicts their behavior in the 'ratio analysis'. Moreover, some genes exhibiting regulatory behavior in the 'ratio analysis' (Fig. 3, lower table half, e.g. Sn-1) do not exhibit local or systemic regulation in the 'state analysis'. All results in which both analyses fail to confirm each other should be interpreted with care.

'State analysis' reveals changes in local *and* systemic expression (e.g. for PIs) which are otherwise not detectable in the 'ratio analysis' since they do not change the sink–source ratio but are only superimposed on it. Only considerably larger changes in expression in either sink or source tissue are detectable as a change in the ratio.

Discussion

A major challenge to the use of microarrays in ecological analysis is the need to have adequate replication within the financial constraints of the study. By using an alternative hybridization scheme (Fig. 1, slides 3 and 4) instead of simply replicating the hybridization scheme represented by slides 1 and 2 we showed that (1) some of the results obtained with both approaches are consistent with each other (which justifies considering the alternative hybridizations as replicate hybridizations) and (2) two different hybridization schemes can be used to provide additional information. For example, a hybridization scheme in which RNA from several treatments is hybridized with the same reference RNA allows these treatments to be compared directly. In our case, hybridizations of RNA from aphid-elicited plants with RNA from insect-free plants (Fig. 1, slides 1 and 2) enabled comparisons with hybridizations of RNA from *M. sexta*- or *T. notatus*-induced plants with RNA from insect-free plants. The analysis of sink–source gene expression ratios in the absence and presence of aphids (Fig. 1, slides 3 and 4) allowed specific hypotheses to be tested, such as whether sink tissues express defense genes differently from source tissues and whether aphid-induced shifts in source-sink expression occur.

In the following text, we discuss (1) how *M. nicotianae* modulates plant gene expression differently from insects of other feeding guilds, (2) whether some of the changes indicate the aphids are manipulating plant metabolism for their benefit, and (3) if the feeding preference of the aphids for sink leaves correlates with differences in the transcription of defense genes between source and sink tissues.

Our microarray analysis revealed that aphid attack elicited the transcriptional signatures characteristic of *M. sexta* and *T. notatus* attack, namely upregulation of defense-related genes and downregulation of photosynthesis-related genes, exemplified by local and systemic upregulation of TPIs and downregulation of the small subunit of Rubisco 48 h after aphid attack. While increases in PI transcripts were not seen in tomato after 1 week of potato aphid feeding (Fidantsef *et al*. 1999), and no information on PI responses is available in the *M. persicae–Arabidopsis* interaction (not tested with the expressed sequences tag (EST) array of Moran *et al*. 2002), greenbug aphid attack increased transcripts of several PI genes in *Sorghum* after 1 d (Zhu-Salzman *et al*. 2004) and *M. euphorbiae/M. persicae* feeding increased PI-I and II transcripts after 6–12 h in two tomato varieties (de Ilarduya *et al*. 2003). Collectively, these results underscore the importance of selecting the 'right' time point in elicitation studies and demonstrate that genes that are commonly associated with the wound response — PIs — are elicited by supposedly 'stealthy' feeders such as aphids. Furthermore, attack from members of all three feeding guilds elicited a common set of genes: increases in LOX- and XTH-expression and a decrease in ubiquitin carrier protein transcripts (genes that were regulated by aphids in the tissues they directly attack). Apart from these similarities, aphids elicited a comparably small transcriptional response, both qualitatively (fewer genes were regulated) and quantitatively (the -fold regulations were smaller). This weak transcriptional response may be a consequence of either the aphid's stealthy feeding behavior or the selection of genes on the array. If this collection of genes had been enriched with aphid-responsive genes (compare the Zhu-Salzman *et al*. 2004 study), a stronger response may have emerged. However, although this array was not enriched in mirid-induced genes, a strong mirid-induced response was nevertheless found (Fig. 2; Voelckel & Baldwin 2004). While this array does not comprehensively characterize a plant's response to attack from any one of the insects, it compares the response of a subset of *M. sexta*-responsive genes to attack from three different herbivore species. Regardless of the array's limitations, an interesting pattern was observed: the up-regulation of glutamate synthase and the down-regulation of germin, a H_2O_2-generating enzyme, in leaves attacked by aphids. This pattern was detected with both hybridization approaches and appears to be unique to the *M. nicotianae–N. attenuata* interaction, since these genes were not similarly induced or repressed by *M. sexta* or *T. notatus* attack. In contrast, glutamate synthase, a gene pivotal in nitrogen assimilation, was down-regulated by *M. sexta* attack.

The up-regulation of glutamate synthase suggests an aphid-induced increase in glutamate production. Glutamate is one of the nitrogen transport molecules in plants and supplies reduced nitrogen for aa synthesis. Interestingly, an increase in tryptophan biosynthesis genes was induced by *M. persicae* in *Arabidopsis* (Moran *et al*. 2002). The upregulation of aa synthesizing genes by aphids could explain the mechanism for a phenomenon observed earlier by Sandstrom *et al*. (2000): the greenbug aphid not only elevated the aa concentration in the phloem sap of wheat and barley, but it also enhanced the proportion of essential aas therein. as validated by stylet exudate- and cut leaf exudate-analyses. This manipulation of phloem-sap composition was interpreted as a means of becoming more independent from bacterial endosymbionts (Sandstrom *et al*. 2000). Phloem aa composition appears to influence the nutritional quality of plants for aphids, as supported in another correlative study in which the potato aphid and the green peach aphid performed better on pretuber-filling potato plants with high glutamine levels than on tuber-filling plants with low glutamine levels (Karley *et al*. 2002). An array containing additional aa metabolism genes may reveal the extent to which aphids manipulate primary metabolism. However, in order to feed efficiently on phloem, aphids not only have to increase the nutritional value of their diet, they must also cope with a plant's constitutive defenses. Therefore, we examined differential transcription of defense genes between potential aphid feeding sites, namely source and sink leaves.

Young leaves had higher Trypsin PI- and HPL-transcript levels than did old leaves, confirming previous results from different (Howe *et al*. 2000; Vancanneyt *et al*. 2001) and the same plant systems (Schittko & Baldwin 2003). These results are in accord with the 'Optimal Defense Theory', which predicts tissues with high fitness values to be best defended (Feeny 1976; Rhoades 1979). C-6 aldehydes derived from HPL-mediated catabolism of 13-hydroperoxides were shown to adversely affect the fecundity of *M. nicotinae/M. persicae* feeding on tobacco leaves exposed to these compounds (Hildebrand *et al*. 1993) or wild-type as opposed to antisense HPL potato plants (Vancanneyt *et al*. 2001). Although PIs are assumed to have little effects on phloem-feeders whose diet contains mainly free aa, PIs from potato increased the mortality of three cereal aphid species (*Diuraphis noxia*, *Schizaphis graminum* and *Rhoalosiphum padi*) in feeding trials (Tran *et al*. 1997). Similarly, Rhabé *et al*. (2003a) found weight and fecundity of *M. persicae* aphids to be reduced on oilseed rape plants that constitutively expressed the cysteine proteinase inhibitor oryzacystatin (OC-1). The deleterious effects of OC-1 correlated with a decrease in cathepsin L/H-type cysteine protease activity in extracts of whole insects. OC-1 itself was not only found

in the digestive tract but it was associated with bacteriocytes, suggesting that OC-1 interacts with the bacterial symbioses which are essential for aphid reproduction (Rahbé *et al*. 2003a). In another study, Bowman-Birk bifunctional trypsin/chymotrypsin inhibitors purified from pea were toxic to pea aphids despite the lack of chymotrypsin activity in aphid guts (Rahbé *et al*. 2003b).

Why do aphids preferentially feed on young leaves that exhibit higher mRNA expression of genes (*HPL* and *PI*) with proven adverse effects on aphid performance? Vancanneyt *et al*. (2001), who found higher HPL transcripts in younger leaves, did not find differences in HPL activity of young compared with old leaves in potato, which suggests that this gene is not under simple transcriptional regulation. The PI activity (which is measured as PI/mg total protein) is reported to be lower in younger than in older *N. attenuata* leaves (J. Zavala and I. T. Baldwin, unpublished results), but these differences likely reflect the greater protein contents of young leaf extracts, rather than true differences in the amounts of PI proteins. However, total leaf protein contents should not be critical for aphid nutrition, and ascertaining whether an increase in PI mRNA levels translates into elevated PI activity in phloem elements, which in turn requires phloem-specific detection of PI proteins, would be more appropriate. Such an analysis has recently been accomplished by Haebel & Kehr (2001), who found PIs in phloem exudates of cucumber. However, PIs and HPL are not the only relevant resistance traits for aphids. For example, Goundoudaki *et al*. (2003) found that aphid performance was positively correlated with leaf sugar levels but negatively correlated with trichome density. Together, these findings underscore the need to analyse mRNA, protein and secondary metabolites levels in the exact tissue types on which aphids feed and contact (i.e. phloem and epidermal cells, and trichomes).

Aphids reproduce quickly and produce large populations on *N. attenuata* plants. Their success likely results from their ability to simultaneously suppress plant defense responses and manipulate phloem flow and composition. This study represents a first step in elucidating the transcriptional mechanisms behind these suppressions and manipulations. Functional studies using knockout plants silenced in lipoxygenase, proteinase inhibitor, xyloglucan-endotransglycosylase, glutamate synthase or germin expression are needed to test the predictions of this study, namely, that the induction of these genes alters the susceptibility of *N. attenuata* to *M. nicotianae* attack and that these genes 'matter' for the interaction.

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References

Arimura G, Tashiro K, Kuhara S *et al*. (2000) Gene responses in bean leaves induced by herbivory and by herbivore-induced volatiles. *Biochemical and Biophysical Research Communications*, **277**, 305–310.

Blackman RL (1987) Morphological discrimination of a tobacco-feeding form from *Myzus persicae* (Sulzer) (Hemiptera: Aphididae), and a key to New World *Myzus* (Nectarosiphon) species. *Bulletin of Entomological Research*, **77** (713), 730.

Cabrera HM, Argandona VH, Corcuera LJ (1994) Metabolic changes in barley seedlings at different aphid infestation levels. *Phytochemistry*, **35**, 317–319.

Clements KM, Wiegmann BM, Sorenson CE *et al*. (2000a) Genetic variation in the *Myzus persicae* complex (Homoptera: Aphididae): evidence for a single species. *Annals of the Entomological Society of America*, **93**, 31–46.

Clements KM, Sorenson CE, Wiegmann BM, Neese PA, Roe RM (2000b) Genetic, biochemical, and behavioral uniformity among populations of *Myzus nicotianae* and *Myzus persicae*. *Entomologia Experimentalis et Applicata*, **95**, 269–281.

Crafts-Brandner SJ, Chu CC (1999) Insect clip cages rapidly alter photosynthetic traits of leaves. *Crop Science*, **39**, 1896–1899.

Feeny P (1976) Plant apparency and chemical defense. *Recent Advances in Phytochemistry*, **10**, 1–40.

Fidantsef AL, Stout MJ, Thaler JS, Duffey SS, Bostock RM (1999) Signal interactions in pathogen and insect attack: expression of lipoxygenase, proteinase inhibitor II, and pathogenesis-related protein P4 in the tomato, *Lycopersicon esculentum*. *Physiological and Molecular Plant Pathology*, **54**, 97–114.

Goundoudaki S, Tsitsipis JA, Margaritopoulos JT, Zarpas KD, Divanidis S (2003) Performance of the tobacco aphid *Myzus persicae* (Hemiptera: Aphididae) on Oriental and Virginia tobacco varieties. *Agricultural and Forest Entomology*, **5**, 285–291.

Haebel S, Kehr J (2001) Matrix-assisted laser desorption/ionization time of flight mass spectrometry peptide mass fingerprints and post source decay: a tool for the identification and analysis of phloem proteins from *Cucurbita maxima* Duch. separated by two-dimensional polyacrylamide gel electrophoresis. *Planta*, **213**, 586–593.

Halitschke R, Gase K, Hui DQ, Schmidt DD, Baldwin IT (2003) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. VI. Microarray analysis reveals that most herbivore-specific transcriptional changes are mediated by fatty acid-amino acid conjugates. *Plant Physiology*, **131**, 1894–1902.

Hermsmeier D, Schittko U, Baldwin IT (2001) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. I. Large-scale changes in the accumulation of growth- and defense-related plant mRNAs. *Plant Physiology*, **125**, 683–700.

Hildebrand DF, Brown GC, Jackson DM, Hamiltonkemp TR (1993) Effects of some leaf-emitted volatile compounds on aphid population increase. *Journal of Chemical Ecology*, **19**, 1875–1887.

Howe GA, Lee GI, Itoh A, Li L, DeRocher AE (2000) Cytochrome P450-dependent metabolism of oxylipins in tomato. Cloning and expression of allene oxide synthase and fatty acid hydroperoxide lyase. *Plant Physiology*, **123**, 711–724.

Hui DQ, Iqbal J, Lehmann K *et al*. (2003) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. V. Microarray

analysis and further characterization of large-scale changes in herbivore-induced mRNAs. *Plant Physiology*, **131**, 1877–1893.

de Ilarduya OM, Xie QG, Kaloshian I (2003) Aphid-induced defense responses in Mi-1-mediated compatible and incompatible tomato interactions. *Molecular Plant–Microbe Interactions*, **16**, 699–708.

Karley AJ, Douglas AE, Parker WE (2002) Amino acid composition and nutritional quality of potato leaf phloem sap for aphids. *Journal of Experimental Biology*, **205**, 3009–3018.

Larson KC, Whitham TG (1997) Competition between gall aphids and natural plant sinks: plant architecture affects resistance to galling. *Oecologia*, **109**, 575–582.

McKenzie CL, Shatters RG, Doostdar H *et al*. (2002) Effect of geminivirus infection and *Bemisia* infestation on accumulation of pathogenesis-related proteins in tomato. *Archives of Insect Biochemistry and Physiology*, **49**, 203–214.

Moran PJ, Cheng YF, Cassell JL, Thompson GA (2002) Gene expression profiling of *Arabidopsis thaliana* in compatible plant–aphid interactions. *Archives of Insect Biochemistry and Physiology*, **51**, 182–203.

Moran PJ, Thompson GA (2001) Molecular responses to aphid feeding in *Arabidopsis* in relation to plant defense pathways. *Plant Physiology*, **125**, 1074–1085.

Pawlowski K, Kunze R, de Vries S, Bisseling T (1994) Isolation of total, poly(A) and polysomal RNA from plant tissue. In: *Plant Molecular Biology Manual* (eds Gelvin SB, Schilperoort RA), pp. 1–4. Kluwer Academic Publishers, Dordrecht.

Rahbe Y, Deraison C, Bonade-Bottino M *et al*. (2003a) Effects of the cysteine protease inhibitor oryzacystatin (OC-I) on different aphids and reduced performance of *Myzus persicae* on OC-I expressing transgenic oilseed rape. *Plant Science*, **164**, 441–450.

Rahbe Y, Ferrasson E, Rabesona H, Quillien L (2003b) Toxicity to the pea aphid *Acyrthosiphon pisum* of anti-chymotrypsin isoforms and fragments of Bowman–Birk protease inhibitors from pea seeds. *Insect Biochemistry and Molecular Biology*, **33**, 299–306.

Reymond P, Weber H, Damond M, Farmer EE (2000) Differential gene expression in response to mechanical wounding and insect feeding in *Arabidopsis*. *Plant Cell*, **12**, 707–719.

Rhoades DF (1979) Evolution of plant chemical defense against herbivores. In: *Herbivores: their Interactions with Plant Secondary Metabolites* (eds Rosethal GA, Jansen DH), pp. 3–54. Academic Press, New York.

Sandstrom J, Moran N (1999) How nutritionally imbalanced is phloem sap for aphids? *Entomologia Experimentalis et Applicata*, **91**, 203–210.

Sandstrom J, Telang A, Moran NA (2000) Nutritional enhancement of host plants by aphids — a comparison of three aphid species on grasses. *Journal of Insect Physiology*, **46**, 33–40.

Schittko U, Baldwin IT (2003) Constraints to herbivore-induced systemic responses: Bidirectional signaling along orthostichies in *Nicotiana attenuata*. *Journal of Chemical Ecology*, **29**, 763–770.

Tran P, Cheesbrough T, Keickhefer R (1997) Plant proteinase inhibitors are potential anticereal aphid compounds. *Journal of Economic Entomology*, **90**, 1672–1677.

Vancanneyt G, Sanz C, Farmaki T *et al*. (2001) Hydroperoxide lyase depletion in transgenic potato plants leads to an increase in aphid performance. *Proceedings of the National Academy of Sciences of the United States of America*, **98**, 8139–8144.

Voelckel C, Baldwin IT (2004) Herbivore-induced plant vaccination. Part II. Array-studies reveal the transience of herbivore-specific transcriptional imprints and a distinct imprint from stress combinations. *Plant Journal*, **38**, 650–663.

Walling LL (2000) The myriad plant responses to herbivores. *Journal of Plant Growth Regulation*, **19**, 195–216.

Zhu-Salzman K, Salzman RA, Ahn JE, Koiwa H (2004) Transcriptional regulation of *Sorghum* defense determinants against a phloem-feeding aphid. *Plant Physiology*, **134**, 420–431.

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