

Systemic response to aphid infestation by *Myzus persicae* in the phloem of *Apium graveolens**

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Abstract

Little is known about the molecular processes involved in the phloem response to aphid feeding. We investigated molecular responses to aphid feeding on celery (*Apium graveolens* cv. Dulce) plants infested with the aphid *Myzus persicae*, as a means of identifying changes in phloem function. We used celery as our model species as it is easy to separate the phloem from the surrounding tissues in the petioles of mature leaves of this species. We generated a total of 1187 expressed sequence tags (ESTs), corresponding to 891 non-redundant genes. We analysed these ESTs *in silico* after cDNA macroarray hybridisation. Aphid feeding led to significant increase in RNA accumulation for 126 different genes. Different patterns of deregulation were observed, including transitory or stable induction 3 or 7 days after infestation. The genes affected belonged to various functional categories and were induced systemically in the phloem after infestation. In particular, genes involved in cell wall modification, water transport, vitamin biosynthesis, photosynthesis, carbon assimilation and nitrogen and carbon mobilisation were up-regulated in the phloem. Further analysis of the response in the phloem or xylem suggested that a component of the response was developed more specifically in the phloem. However, this component was different from the stress responses in the phloem driven by pathogen infection. Our results indicate that the phloem is actively involved in multiple adjustments, recruiting metabolic pathways and in structural changes far from aphid feeding sites. However, they also suggest that the phloem displays specific mechanisms that may not be induced in other tissues.

Abbreviations: CMV, cucumber mosaic cucumovirus; EST, expressed sequence tag; JA, jasmonic acid; PR, pathogenesis-related; SA, salicylic acid

Introduction

The co-evolution of phytophagous insects and of the plants they feed on, in terms of responses to insect attack and the mechanisms underlying the recognition, signalling and protection against such attacks, has resulted in a diversity of plant – herbivore defence strategies (Walling, 2000;

*EST, macroarray and clustering data are available from our website [<http://www-biocel.versailles.inra.fr/phloem>]. Data deposition: The sequences reported in this paper have been deposited in the Genbank database (Accession nos.: AY607692-AY607700, AY611007, CN253939-CN255151, CV512445-CV512447 and CV651120-CV651121).

Reymond *et al.*, 2000; Baldwin *et al.*, 2001). Plants synthesise specific compounds, such as volatile compounds and secondary metabolites, but have also developed general mechanisms of defence against insects, fine-tuning the balance between limiting pest multiplication and basic requirements for plant growth. Cross-talk between various defence and metabolic or hormonal pathways provides evidence for the global co-ordination of gene responses (Gatehouse, 2002; Kessler and Baldwin, 2003; Taylor *et al.*, 2004). For example, many plant genes are differentially regulated following infestation with chewing insects, including markers of the wound-response pathway and metabolic regulatory genes (Reymond *et al.*, 2000; Hui *et al.*, 2003; Voelckel and Baldwin, 2003; Zhang *et al.*, 2004). In these studies, the whole-plant response was assessed, either locally or systemically. This whole-plant response reflected a combination of multiple levels of responses, including primary responses in damaged cells and downstream responses in other tissues or organs.

The interaction of plants with phloem-feeding insects, such as aphids, represents an elegant case of specialisation. The development of aphid populations on plants frequently leads to changes in various phloem functions, including nitrogen recycling, as shown by changes in the amino acid content of phloem sap following aphid feeding (Sandström *et al.*, 2000, Gironse *et al.*, 2003) and developmental switches (Gironse *et al.*, 2003). The physiology of the plant may affect these changes, as nitrogen supply and water status have a significant effect on aphid behaviour (Ponder *et al.*, 2001; Hale *et al.*, 2003). The plant–aphid interaction involves three main steps, which determine whether the plant is susceptible or resistant: (1) probing of the cell surface by the aphid, (2) probing of the various cell layers to reach the vascular bundles, (3) initiation of feeding by direct pumping of the phloem sap in the sieve elements. The insects produce a “salivary sheath” to protect their stylets during the initial exploration of the surface cell layers (Miles, 1999). This sheath extends into the apoplasm of the injured cells from the cuticle, through the mesophyll and finally into the phloem. When the insects eventually establish sustained feeding within the sieve elements, they take up large volumes of phloem sap, which may have a strongly deleterious effect on plant growth and development. Strikingly, the

sieve elements do not become plugged during feeding, despite direct damage to the conducting elements. This suggests that components of the aphid saliva injected into the sieve elements prevent these elements from becoming plugged when injured; this plugging is thought to involve the deposition of callose on the sieve plates or the aggregation of P-proteins (Miles and Oertli, 1993). The saliva of aphids also contains various hydrolytic enzymes (Miles, 1999) that may elicit plant defences or pre-empt potential wound responses. The saliva compounds released into the phloem are complex and little is known about their precise functions (Miles, 1999; Cherqui and Tjallinghii, 2000), although some elements of the plant response have recently been identified. Moran and Thompson (2001) analysed the molecular response of *Arabidopsis thaliana* to *Myzus persicae*, and demonstrated the existence of a rapid local defence response in infested leaves. This response involved the jasmonic acid (JA) and salicylic acid (SA) pathways. These results were also supported by genetic evidence: Ellis *et al.* (2002) showed that plants with modified JA pathways were more resistant to aphids than wild-type plants. However, the local defence response was not followed by the systemic induction of defence genes associated with wounding and pathogen attacks (Moran and Thompson, 2001). This observation provided evidence to suggest that aphids are able to block the generalised defence response to some extent, despite feeding directly on phloem sap. A more detailed analysis recently described the complexity of the response to aphid infestation in *A. thaliana* (Moran *et al.*, 2002), *Sorghum bicolor* (Zhu-Salzman *et al.*, 2004) and *Nicotiana attenuata* (Voelckel *et al.*, 2004). These reports suggested that aphids might be able to avoid activating potentially effective defensive machinery. In these studies, the response in the phloem, the primary target of aphid feeding, was not distinguished from total organ or plant responses. The question of the primary steps in the response initiated in the phloem therefore remained unanswered.

The phloem constitutes a small fraction of the tissues of the plant (< 0.5% of total leaf volume; Sjölund, 1997). The response of this vascular tissue is therefore likely to be masked by those of the surrounding tissues. As the phloem is an important control centre for long-distance signalling and

carbohydrate partitioning (Van Bel, 2003), the systemic plant response, including metabolic readjustments and defence signalling, is thought to be triggered from the phloem. We aimed to dissect this response during aphid feeding by investigating specific changes in this tissue. Various methods are now available for separating vascular cell layers from the surrounding cells (Hertzberg *et al.*, 2001; Asano *et al.*, 2002; Nakazono *et al.*, 2003; Ivashikina *et al.*, 2003; Birnbaum *et al.*, 2003). We chose to study celery (*Apium graveolens*), because the peeling of the petioles to separate the phloem from the surrounding tissues is a simple and reliable method for the separation of vascular tissues. This technique has been used to identify, by transcript profiling, sets of genes preferentially expressed in the phloem (Vilaine *et al.*, 2003). As the aphids that feed on leaf blades do not usually colonise the petioles used for phloem separation, it was possible to follow the systemic phloem response. Phloem-enriched tissue fractions from infested plants were used to identify genes differentially expressed in the phloem tissue in response to aphid infestation, by means of expressed sequence tag (EST) sequencing and cDNA macroarray hybridisation.

Results

Aphid infestation of celery plants

Celery is a natural host for the green peach aphid (*M. persicae*). Four-month-old celery (*A. graveolens* var. Dulce) plants were infested with *M. persicae*, using clonal populations acclimated and grown on celery plants. Three days post-infestation (dpi), aphid populations were well established and successfully feeding on fully expanded mature leaves. Significant colonisation of the plant 7 dpi was defined as the presence of multiple colonies on most of the leaflets of mature leaves, indicating that the aphids were reproducing. This resulted in massive infestation of the plants, with hundreds of insects developing on the abaxial side of mature leaves. Interestingly, young and immature leaves, close to the plant apex, remained free of insects. Three weeks after infestation, the plant showed symptoms of early senescence, with advanced yellowing of the older

leaves and growth reduction. No hypersensitive response was observed at the feeding sites. The vascular tissues of infested plants appeared normal.

Analysis of the sequences of randomly selected cDNAs

Phloem and xylem strands from detached petioles were separated from the surrounding tissues containing collenchyma, cortical and medullar storage parenchyma and epiderm (hereafter referred to collectively as “parenchyma”). The phloem was then separated from the xylem and used to prepare phloem-enriched cDNA libraries. These libraries were enriched for stress-response sequences by subtractive suppression hybridisation (SSH) (Diatchenko *et al.*, 1996) between cDNAs from the phloem of infested and non-infested plants. Individual clones were randomly selected for cDNA sequencing and macroarray spotting.

Single-pass sequences were obtained from 1150 clones from a cDNA library enriched with up-regulated genes. We also obtained 37 ESTs from another cDNA library (BRP) generated using reverse conditions for SSH to enrich the library in down-regulated genes. We obtained 891 contiguous sequences from the 1187 ESTs studied as 25% of the sequences were redundant. These sequences were represented once (82%), twice (12%) or three times or more (6%), with a maximum of 26 copies for the celery phloem-specific gene *AgNS1*, previously described by Vilaine *et al.* (2003), which belongs to a class of genes of unknown function displaying no similarity (NS for no score) to sequences from other species. As these ESTs correspond to partial cDNAs, we could not exclude the possibility that some non-overlapping contigs covered individual genes.

The ESTs were annotated by means of BLAST similarity searches, which revealed significant matches (threshold E-value = 10^{-5}) with genes of assigned function for 51% of the sequences (Supplementary Materials 1). This value is consistent with that obtained in a previous phloem EST analysis of a SSH cDNA library (45% annotated sequences; Vilaine *et al.*, 2003). ESTs were classified into the main functional categories and the distribution obtained was similar to that for phloem cDNA libraries not enriched in aphid-induced sequences (Figure 1). The ESTs derived

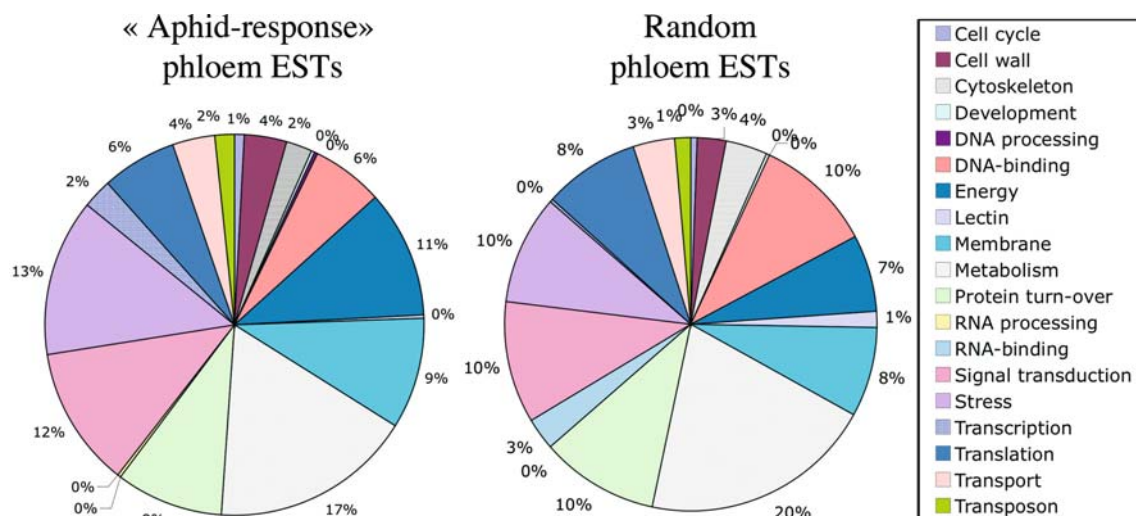


Figure 1. Distribution of ESTs according to functional classes. "Aphid-induced" ESTs: ESTs from the phloem subtraction cDNA library generated from the petioles of aphid-infested plants in this study. Standard ESTs: ESTs from standard phloem cDNA libraries generated from young or mature petioles as described by Vilaine *et al.*, (2003).

from infested plants were only moderately redundant (10%) with ESTs obtained in standard growth conditions (Vilaine *et al.*, 2003). We were unable to annotate 48% of the sequences: 20% corresponded to sequences similar to annotated genes of unknown function and 28% did not match other sequences.

More detailed examination of the functions represented in these libraries showed differences in representation of the more specific functions (Table 1). Genes with functions related to carbohydrate partitioning and metabolism and nitrogen recycling were more frequent in the cDNA library generated from aphid-infested plants, as were several genes frequently associated with oxidative stress (metallothioneins, catalases, peroxidases). Genes encoding phloem-specific proteins that appeared stably expressed in that tissue such as the phloem-specific lectin, AgPP2-1, the AgCAT1 and AgCAT2 catalases, the nodulin-like AgNOD1, and the OleE1 allergen (Dinant *et al.*, 2003; Vilaine *et al.*, 2003) were poorly represented, showing that subtraction eliminated the sequences corresponding to such proteins.

*Cloning of additional control markers in *Apium graveolens**

In addition to the cDNA clones generated by SSH representing a subpopulation of genes differentially expressed in response to aphid infestation,

other cDNA clones were collected for use as internal controls in macroarray experiments. These cDNAs corresponded to putative "house-keeping" genes, expressed equally strongly in all tissues and conditions, and markers reflecting the physiological, metabolic and stress status of the plants. These cDNAs included genes for which mRNA was produced constitutively in experiments in various petiole tissues (clones defined as "stable" in Vilaine *et al.*, 2003), such as *Eif4A*, *Eef1a* and a profilin gene. As the principal aim was to analyse phloem response, cDNA clones corresponding to genes preferentially expressed in the phloem tissue and only slightly affected by physiological conditions were also included (*AgPP2-1*, an ubiquitin-conjugating enzyme gene *E2* and the nodulin-like *AgNod1* gene). We also included genes strongly expressed in the vascular tissues, such as those encoding the metallothioneins *AgMT1*, *AgMT2* and *AgMT4* (Vilaine *et al.*, 2003). Several clones for metabolic markers and membrane transporters were also included: a methionine synthase, the mannitol transporter *AgMAT1*, the sucrose transporter *AgSUT2*, a vacuolar invertase and the mannose-6-phosphate dehydrogenase (Lemoine, unpublished results; Noiraud *et al.*, 2000, 2001).

Finally, this set included several putative defence genes, because genes corresponding to the general defence response were underrepresented in this set of ESTs. As little is known about the

Table 1. Number and frequency of the most abundant celery ESTs found in the subtracted phloem BIP cDNA library enriched in sequences responding to aphid infestation. For comparison, the frequency of these ESTs in standard phloem cDNA libraries, as described by Vilaine *et al.* (2003), are indicated.

Function	Putative identity	Accession no.	Number [and frequency; %] of ESTs in BIP cDNA library (1150 EST)	Number [and frequency; %] of ESTs in standard cDNA libraries (721 EST)
Metabolism/nitrogen recycling	Glutamine synthetase GS1	CN254940	5 [4.3]	0 [0]
	Mannitol transporter	CN254621	3 [5.1]	0 [0]
	AgMAT1	CN254915	3	
	Sucrose synthase Susy	CN255033	3 [2.6]	0 [0]
	Sucrose transporter AgSUT2	BU693157	0 [0]	2 [1.7]
	Peptide transporter	CN254498	4 [3.4]	0 [0]
	Thiamine biosynth. enz Thi1-1	CN254190	3 [2.6]	1 [0.9]
	Thiamine biosynth. enz Thi1-2	CN254452	5 [4.3]	1 [0.9]
	β -galactosidase	CN254209	4 [3.4]	0 [0]
Structure	Lectin AgPP2-1	CN254691	1 [0.9]	4 [3.4]
Stress response	Catalase AgCAT1	CN254916	1 [0.9]	12 [10.2]
	Copper homeostasis factor CCH	CN254148	4 [3.4]	0 [0]
	Dehydrin ecpp44	CN254230	5 [4.3]	0 [0]
	Metallothionein AgMT1	CN254988	4 [3.4]	1 [0.9]
	Metallothionein AgMT2	CN254899	4 [3.4]	3 [2.6]
	Metallothionein AgMT3	CN254033	6 [5.1]	2 [1.7]
	Metallothionein AgMT5	CN254935	5 [4.3]	0 [0]
	Aluminium-induced ARG10	BU693589	0 [0]	4 [3.4]
	Peroxidase	CN254785	9 [7.6]	0 [0]
Energy	Photosystem II, LS1	CN254833	3 [2.6]	1 [0.9]
	Cytochrome c1	CN254568	3 [2.6]	0 [0]
	CAB (cab-11)	CN254590	1 [0.9]	5 [4.3]
	CAB (cab-2)	BU693425	0 [0]	2 [1.7]
Signal transduction	Ethylene-responsive element	CN254275	3 [2.6]	0 [0]
	Giberellin GAST1	CN254272	8 [6.8]	0 [0]
	Ethylene-responsive EREBP-3	BU693337	0 [0]	3 [2.6]
Translation and transcription	Translation factor eEF1- α	BU693243	0 [0]	2 [1.7]
	Translation factor eEF1- α	CN254794	3 [2.6]	1 [0.9]
	Dead box RNA helicase	CN254133	4 [3.4]	0 [0]
	Homeobox leucine zipper	CN254901	3 [2.6]	0 [0]
	Transcription factor RAU1	CN254584	3 [2.6]	0 [0]
	DNA binding HAT7	BU693245	0 [0]	3 [2.6]
Miscellaneous	Aquaporin PIP1	CN254401	4 [3.4]	0 [0]
	Ubiquitin-conjugating enz E2	BU693414	0 [0]	3 [4.3]
		BU693408	0	2
Unknown function	Unknown AgNS1	CN255026	26 [22]	1 [0.9]
	Unknown AgNS4	CN254155	11 [9.3]	0 [0]
	Unknown AgUNK5	CN254299	5 [4.3]	0 [0]
	Major allergen PR10	CN254442	12 [10.2]	1 [0.9]
	Allergen ole e1	CN254402	1 [0.9]	4 [3.4]

response of celery to pathogen attack or stress, partial cDNAs corresponding to putative celery orthologues of defence genes well characterised in other species, associated with wounding or pathogen attacks, were amplified with degenerate primers deduced from conserved regions found in their sequences (Table 2) and cloned. These sequences encoded the putative orthologues of two pathogenesis-related (PR) proteins, PR2 and PR10, and enzymes involved in octadecanoid, flavonoid and phenolic pathways frequently induced by biotic stresses. We also cloned two additional markers, *MPK4* (Petersen *et al.*, 2000) and *EDR1* (Frye *et al.*, 2001) encoding the putative orthologues of the MAP kinases (Table 2) involved in the signalling cascades triggered in the JA and SA pathways, respectively, in *A. thaliana*.

cDNA macroarray analysis of the expression profile of genes expressed in the phloem

We investigated the pattern of RNA production in the phloem of infested plants, and the expression profile of the additional marker sequences, by cDNA macroarray analysis. Nylon macroarrays were prepared with PCR products corresponding to 1278 cDNA clones, including the 1187 sequenced cDNA clones and the control markers (including those presented in Table 2). The products of PCR with cloning vectors were spotted as negative controls. Each clone was arrayed in duplicate. The accumulation of mRNAs in phloem isolated from the petioles of mature, aphid-infested and control uninfested celery leaves was monitored by hybridisation with specific probes.

Three experiments were performed with independent sets of plants, the infestation of which was monitored 3 or 7 dpi. We isolated tissues at the same time of day, by the same procedure, taking the same time in each case, to minimise physiological variation. The reproducibility of macroarray analysis for scoring differential gene expression has been validated for this system (Vilaine *et al.*, 2003). During normalisation, we carefully took into account bias introduced by enriching libraries with tissue-specific sequences. Significant inter-experiment variation was observed in the transcript profiles for most genes, consistent with the results obtained in a previous study (Vilaine *et al.*, 2003). This variation probably reflects some differences in the physiological status of the plants

between experiments, as plants were grown in greenhouses over a period of 4 months at different periods of the year.

We used analysis of variance (ANOVA) to discriminate between variations due to aphid infestation from those due to other causes (physiological or methodological). This method is particularly suitable for correction for potential confounding effects in macroarray studies (Kerr *et al.*, 2000). This made it possible to identify genes displaying consistent transcript profile variations due to aphid infestation. Almost 40% of the sequences displayed variations in transcript profile for at least one time point (3 or 7 dpi), depending on stress conditions, with P values associated with the condition (P_{valCond}) below 0.01 (Supplementary Materials 2). The magnitude of the changes observed was nevertheless moderate, with the level of expression of many sequences changing by a factor of only 1.2–1.5, suggesting that this change may not be highly relevant biologically. We retained only those genes for which expression level changed by a factor of at least 1.7 on average for subsequent analysis. This threshold far exceeds the error limit calculated for replicate variability (1 ± 0.3) and has been reported to be a good indicator of differential expression for this array technology (Vilaine *et al.*, 2003), despite probably underestimating the actual variations in gene expression. Based on these two criteria (change by a factor > 1.7 , $P_{\text{valCond}} < 0.01$) we retained 202 sequences, grouped into 126 non-redundant contigs (Table 3). These sequences included genes involved in a variety of biological functions, such as cell structure (cell wall, cytoskeleton, membrane), metabolism (carbon assimilation and nitrate or sugar assimilation), protein turnover and stability, oxidative stress, metal homeostasis, stress response, signal transduction and gene expression (translation and DNA-binding proteins). Fifty-one of the 126 non-redundant contigs (41%) corresponded to genes encoding proteins of unknown function found in other species (unknown function: *Unk* genes) or presenting no similarity to any other known sequence (no score: *NS* genes).

Transcription profile of genes up-regulated after infestation

Cluster analysis by hierarchical procedures identified three main subgroups of expression pattern for up-regulated genes in infested and non-infested

Table 2. List of celery metabolic and stress marker cDNAs spotted on the cDNA macroarray in addition to BIP cDNA clones.

Clone	Putative identity	Accession no.	Homologue*
General defence response			
CTL066	Lipoxygenase	CB275388 (1)	-
CTL040	Lipoxygenase LOX1	AY607693 (7)	Q06327 (2) [<i>Arabidopsis thaliana</i>]
CTL038	Chalcone synthase CHS	AY607692 (7)	M20308 (4) [<i>Arabidopsis thaliana</i>]
CTL041	MAP kinase kinase kinase EDR1	AY607694 (7)	AAG31143 (5) [<i>Arabidopsis thaliana</i>]
CTL042	PR protein PR10 (ApiG1)	BU693177 (1)	D88388 (6) [<i>Daucus carota</i>]
CTL044	MAP kinase MPK4	AY607695 (7)	D21840 (8) [<i>Arabidopsis thaliana</i>]
CTL046	Hevein-like protein HEL	AY607696 (7)	U01880 (10) [<i>Arabidopsis thaliana</i>]
CTL047	14-3-3 Protein	AY607697 (7)	X74141(11) [<i>Arabidopsis thaliana</i>]
CTL048	PR2 (β -1-3 glucanase BGL2)	AY607698 (7)	X58698 (12) [<i>Petroselinum crispum</i>]
CTL049	Caffeoyl-CoA 3-O-methyltransferase	AY607699 (7)	M69184(13) [<i>Petroselinum crispum</i>]
CTL050	Fatty acid desaturase ELI7.1	AY607700 (7)	AF239827 (14) [<i>Petroselinum crispum</i>]
CTL059	Protease inhibitor IP2	BU693794 (1)	-
Metal homeostasis-redox potential			
CTL055	Metallotionein AgMT1	BU693865 (1)	-
CTL058	Metallotionein AgMT2	BU693948 (1)	-
CTL062	Metallotionein AgMT4	BU693877 (1)	-
Nitrogen recycling			
CTL045	Glutamine synthetase GS2	AY611007 (7)	AF019561 (9) [<i>Daucus carota</i>]
Carbohydrate allocation and metabolism			
CTL093	Sucrose synthase Susy	AF393809 (3)	-
CTL089	Sucrose transporter SUT2	AF167415 (16)	-
CTL039	Mannitol transporter MAT1	AF215837 (15)	AF215837 (15) [<i>Apium graveolens</i>]
CTL091	Mannose-6-phosphate reductase	U83687 (17)	-
CTL092	Mannitol dehydrogenase	Q38707 (18)	-
CTL088	Vacuolar invertase	AF393810 (3)	-
Metabolism			
CTL094	Glyceraldehyde-3-phosphate dehydrogenase (G3PDH)	AF196292 (1)	-
CTL029	S-adenosyl methionine synthetase SAMS1	BU693107 (1)	-
CTL031	S-adenosyl methionine synthetase SAMS2	BU693407 (1)	-
CTL002	S-adenosyl methionine decarboxylase	BU693146 (1)	-
Cytoskeleton			
CTL016	Actin	BU693206 (1)	-
CTL019	Actin depolymerising factor	BU693025 (1)	-
CTL024	α -tubulin	BU693907 (1)	-
CTL027	α -tubulin	BU693241 (1)	-
CTL025	Profilin ApiG4	BU693489 (1)	-
Miscellaneous			
CTL085	Histone H3.3	BU693854 (1)	-
CTL011	Aquaporin - water channel	BU693400 (1)	-
CTL077	Ubiquitin-conjugating enzyme E2	CB275394 (1)	-
CTL051	Eef1 α	BU693199 (1)	-
CTL096	Eif4A	AF393811 (3)	-

*Homologue: For the new sequences amplified by RT-PCR and presented in this work, the sequence of the type member chosen for the identification of a conserved polynucleotide used for the design of degenerate primers.

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Table 3. List of the 126 non-redundant genes showing the strongest upregulation following infestation with aphids (Pval_{Cond}1.7 at 3 or 7 dpi).

Function	Clone	PUTATIVE IDENTITY	Accession no.	Response at 3 dpi PVAL _{CON} (AVERAGE RATIO)	Response at 7 dpi PVAL _{COND} (AVERAGE RATIO)
Cell wall	BIP0766	Cellulose synthase [171]	CN254570	2.8E-04 (1.94 ± 0.5)	7.8E-03 (1.43 ± 0.2)
	BIP0008	Expansin	CN253946	6.1E-03 (1.86 ± 1)	1.8E-01 (1.63 ± 1.3)
	CTL010	Expansin	BU693438	6.9E-03 (1.91 ± 0.9)	2.0E-01 (1.34 ± 0.8)
	BIP0289	Pectin acetyltransferase	CN254169	1.1E-03 (1.92 ± 0.5)	1.2E-01 (1.11 ± 0)
	BIP1226	Pectinesterase	CN254944	6.6E-05 (1.77 ± 0.3)	1.4E-02 (1.27 ± 0)
	BIP0619	Pectinesterase	CN254453	9.1E-03 (1.8 ± 1.1)	3.2E-01 (1.13 ± 0.1)
	BIP0760	Xyloglucan endotransglycosylase [*]	CN254566	1.4E-01 (1.24 ± 0.1)	6.8E-04 (2.49 ± 2.7)
	BIP1089	Xyloglucan endotransglycosylase	CN254829	7.0E-04 (2.09 ± 0.6)	8.1E-01 (0.99 ± 0.1)
	BIP0934	Xyloglucan endotransglycosylase [257]	CN254701	5.3E-04 (2 ± 0.3)	4.3E-03 (1.31 ± 0.1)
	BIP0863	Actin-depolymerising factor 2 [200]	CN254642	7.0E-05 (1.72 ± 0.1)	7.9E-03 (1.29 ± 0.1)
Cytoskeleton	CTL024	Alpha-2 tubulin	BU693907	9.5E-04 (1.73 ± 0.4)	2.0E-01 (1.63 ± 1.4)
	BIP0989	Beta-tubulin	CN254749	4.2E-03 (1.38 ± 0.1)	2.4E-05 (1.75 ± 0.2)
	CTL014	Microtubule	BU693451	2.7E-04 (1.33 ± 0)	1.2E-04 (1.75 ± 0.2)
	BIP0463	Tubulin alpha-6 chain	CN254316	9.7E-04 (2.16 ± 0.8)	8.3E-01 (1.2 ± 0.3)
	BIP1230	Tubulin beta 1 [223]	CN254947	1.2E-06 (2.56 ± 1)	2.6E-01 (1.03 ± 0.1)
	CTL033	DNA-binding	BU693897	6.0E-06 (2.28 ± 0.5)	1.1E-01 (1.18 ± 0.1)
	CTL085	Histone H3.3	BU693854	5.3E-03 (3.96 ± 14.6)	1.3E-01 (1.71 ± 1.5)
	BIP1177	Homeobox-leucine zipper [218]	CN254901	8.8E-01 (1.1 ± 0.3)	3.4E-03 (2.34 ± 1.7)
	BIP0205	Nucleoid DNA-binding protein	CN254107	2.7E-01 (1.1 ± 0.4)	2.4E-05 (1.9 ± 0.3)
	BIP1219	Aquaporin PIP3	CN254937	3.9E-03 (0.85 ± 0.1)	2.1E-02 (3.74 ± 11.3)
Membrane	BIP0975	Major intrinsic protein (MIP) [267]	CN254738	5.4E-01 (1.25 ± 0.3)	3.5E-04 (2.18 ± 1.2)
	CTL036	Plasma membrane protein	BU693136	2.0E-01 (1 ± 0.3)	3.0E-07 (1.79 ± 0.1)
	BIP0687	Transmembrane protein TIP1.3[*]	CN254506	3.4E-05 (1.84 ± 0.1)	0.0E + 00 (1.8 ± 0.2)
Carbon assimilation	BIP1020	Carbonic anhydrase	CN254776	1.4E-05 (1.97 ± 0.2)	1.2E-03 (1.42 ± 0.1)
	BIP0910	G3PDH [190]	CN254679	2.0E-02 (1.54 ± 0.1)	8.3E-03 (1.92 ± 1.7)
	BIP1017	G3PDH	CN254773	8.2E-01 (1.12 ± 0.3)	3.6E-03 (1.92 ± 2.1)
	BIP0736	Glycolate oxidase [165]	CN254546	2.5E-06 (1.71 ± 0.1)	2.9E-03 (1.9 ± 0.8)
	BIP0467	Thiamine biosynthesis thil-2 [137] [*]	CN254452	1.0E-07 (4.07 ± 6.5)	7.7E-03 (1.26 ± 0)
Metabolism	CTL038	Chalcone synthase CHS2	AY607692	2.8E-04 (2.51 ± 2)	7.5E-02 (1.19 ± 0.1)
	CTL031	S-adenosylmethionine synthetase 1	BU693407	0.0E + 00 (2.62 ± 1)	8.7E-02 (0.86 ± 0)
	CTL029	S-adenosylmethionine synthetase 2	BU693107	1.7E-03 (2.2 ± 0.6)	1.2E-01 (1.15 ± 0)
Metal homeostasis	BIP0634	Copper homeostasis factor [153] [*]	CN254148	1.6E-06 (2.34 ± 0)	1.1E-01 (1.24 ± 0.2)

	CTL062	Metallothionein AgMT4	BU693877	2.2E-03 (2.15 ± 2)	9.2E-02 (1.68 ± 1.2)
	BIP1217	Metallothionein AgMT5 [*]	CN254935	3.2E-05 (2.04 ± 0.4)	3.8E-02 (1.43 ± 0.4)
	BIP1372	Metallothionein	CN255059	1.5E-04 (1.56 ± 0.2)	9.1E-03 (2.15 ± 2)
	BIP0795	Chlorophyll a-b binding protein	CN254590	1.8E-01 (1.01 ± 0.1)	1.6E-04 (2.3 ± 1.3)
	BIP0274	Chlorophyll a-b binding protein	CN254157	3.2E-04 (1.56 ± 0.1)	9.1E-03 (1.97 ± 1.6)
	BIP0546	Cytochrome b6	CN254386	2.6E-01 (1.9 ± 5.8)	5.4E-03 (1.22 ± 0)
	BIP0955	Photosystem I reaction centre [274]	CN254720	1.2E-05 (1.73 ± 0.2)	2.6E-03 (1.49 ± 0.2)
	BIP0950	Photosystem II 10 kDa protein [269]	CN254715	1.5E-01 (0.95 ± 0.2)	2.8E-03 (1.85 ± 0.8)
	BIP1094	Photosystem II LSI protein [208]	CN254833	4.5E-01 (2.41 ± 10.6)	3.8E-04 (1.94 ± 0.6)
	BIP0966	Phytochrome-associated PAP2	CN254730	2.1E-02 (1.28 ± 0.1)	6.0E-04 (2.37 ± 1.1)
	BIP0864	Ribulose carboxylase	CN254643	1.5E-06 (2.51 ± 1)	3.0E-03 (1.26 ± 0)
	BIP1065	Ribulose biphosphate carboxylase	CN254815	1.5E-02 (1.54 ± 0.5)	2.3E-03 (1.8 ± 0.9)
	BIP0979	Ribulose biphosphate carboxylase	CN254741	2.3E-04 (1.86 ± 0.3)	3.0E-02 (1.51 ± 0.6)
	CTL064	Chlorophyllase CLH2	CB275394	1.0E-07 (1.67 ± 0)	1.0E-02 (2.14 ± 2.4)
	BIP0942	Cyclophylin [240]	CN254708	4.4E-05 (1.83 ± 0.2)	5.4E-01 (0.95 ± 0)
	BIP0754	Cyclophylin	CN254561	8.6E-04 (1.75 ± 0.5)	1.0E-03 (1.97 ± 1.1)
	BIP0834	Cysteine proteinase [179]	CN254619	6.6E-02 (1.34 ± 0.2)	6.3E-04 (1.82 ± 0.5)
	CTL077	Ubiquitin-conjugating enzyme E2	CB275394	8.1E-03 (1.31 ± 0.1)	4.5E-06 (2.29 ± 0.6)
	BIP0956	Auxin-regulated protein [207]	CN254721	6.3E-01 (1.4 ± 0.3)	3.7E-04 (2.09 ± 0.7)
	BIP0230	Calmodulin	CN254123	2.4E-04 (2.01 ± 1.9)	5.7E-03 (1.47 ± 0.2)
	CTL007	Ethylene-responsive element	BU693277	8.0E-03 (1.45 ± 0.2)	0.0E + 00 (1.84 ± 0)
	BIP1247	14-3-3 protein-binding	CN254957	3.7E-04 (1.63 ± 0)	5.1E-03 (1.75 ± 0.6)
	BIP1144	Protein kinase	CN254872	3.0E-01 (0.98 ± 0.1)	3.0E-03 (2.1 ± 1.6)
	BIP0850	Cytokinin- binding PR10	CN254631	7.8E-04 (1.79 ± 0.2)	5.2E-01 (1.01 ± 0)
	BIP0880	Snapkin / GAST [*]	CN254656	1.1E-05 (1.97 ± 0.3)	2.0E-04 (1.67 ± 0.2)
	BIP0051	Defensin AMP1	CN253987	6.3E-01 (1 ± 0.1)	5.5E-04 (4 ± 7.5)
	BIP0605	Major allergen Apig1 PR10 [154]	CN254442	3.7E-03 (1.26 ± 0.2)	3.4E-03 (1.74 ± 2.1)
	BIP0994	Phytochelatinsynthetase [197]	CN254754	4.2E-02 (1.37 ± 0.3)	1.1E-02 (2.12 ± 1.9)
	BIP0307	Ascorbate oxidase PS60	CN254184	1.1E-05 (1.93 ± 0.2)	3.0E-02 (1.34 ± 0.2)
	CTL004	Catalase AgCAT1	BU693953	7.4E-03 (1.66 ± 0.8)	1.3E-03 (1.74 ± 0.6)
	BIP0731	Catalase AgCAT2 [164]	CN254544	3.1E-04 (1.63 ± 0)	1.2E-01 (2.88 ± 6.3)
	BIP1208	Glutathione peroxidase	CN254927	5.2E-06 (2.95 ± 2)	2.7E-03 (1.69 ± 1)
	BIP1030	Peroxidase AgPerOx [162]	CN254785	3.1E-04 (1.86 ± 0.2)	7.6E-01 (0.95 ± 0)
	BIP1136	Mannitol/CAD dehydrogenase	CN254867	5.0E-06 (2.02 ± 0.2)	2.7E-02 (1.21 ± 0.1)
	BIP1222	Glutamine synthetase GSI [*]	CN254940	3.7E-01 (1.15 ± 0)	0.0E+00 (1.79 ± 0)

Photosynthesis

Protein turnover

Signal transduction

Stress

Oxidative stress

C and N mobilisation

Table 3. (Continued).

Unknown	BIP0839	Unknown Leucine zipper PNIL34	CN254623	3.4E-03 (2.18 ± 1.7)	2.4E-01 (1.22 ± 0.2)
Translation	CTL065	Mannitol dehydrogenase	BU693942	1.6E-06 (1.72 ± 0)	5.9E-04 (1.44 ± 0.1)
	BIP1191	Mannitol transporter AgMAT1 [241]	CN254915	2.4E-03 (1.78 ± 1)	7.2E-02 (1.26 ± 0.2)
	BIP0574	Nitrate transporter	CN254412	8.5E-03 (0.83 ± 0.1)	6.6E-02 (2.03 ± 1.9)
	BIP0881	Sorbitol dehydrogenase	CN254657	9.0E-02 (1.24 ± 0.1)	7.4E-03 (2.06 ± 1.5)
	BIP0095	40S ribosomal protein	CN254024	1.7E-05 (1.86 ± 0.3)	7.2E-03 (1.35 ± 0.1)
	BIP1452	40S ribosomal protein S13 [247]	CN255113	1.6E-04 (1.75 ± 0.3)	9.3E-03 (2.64 ± 5.4)
	BIP0348	60S ribosomal protein L5	CN254213	1.3E-04 (1.82 ± 0.4)	7.3E-01 (1.11 ± 0.5)
	BIP0724	Poly (A)-binding protein	CN254538	5.4E-04 (1.33 ± 0)	1.4E-03 (2.3 ± 1.8)
	CTL052	Translation factor eIF-4a	BU693942	2.2E-04 (1.4 ± 0.1)	2.8E-03 (1.92 ± 1.6)
	BIP0918	ADP-ribosylation factor [258]	CN254685	2.7E-03 (1.5 ± 0.4)	2.1E-03 (2.12 ± 3.6)
	BIP0372	Unknown dehydrin ecpp44 [125] [*]	CN254230	2.1E-04 (1.75 ± 0.5)	7.6E-01 (1.32 ± 0.8)
	BIP0738	Unknown wound-induced	CN254548	5.2E-04 (1.83 ± 0.6)	5.5E-03 (1.35 ± 0.1)
	BIP0516	Unknown AgUNK3 [*]	CN254362	1.8E-06 (2.32 ± 0.6)	1.0E-01 (0.92 ± 0.1)
Transport	BIP0280	Unknown AgUNK2 [*]	CN254161	6.6E-01 (1.01 ± 0)	0.0E+00 (2.41 ± 0.2)
	BIP0245	Unknown AgUNK4 [213]	CN254135	1.2E-03 (0.84 ± 0.1)	4.9E-06 (2.21 ± 1)
	BIP0219	Unknown pollen surface protein [255]	CN254117	4.5E-03 (1.83 ± 0.5)	7.9E-01 (1.06 ± 0.1)
	BIP0506	Unknown pollen surface protein	CN254352	6.8E-06 (1.74 ± 0.1)	1.9E-03 (0.81 ± 0)
	BIP0563	Unknown major allergen Ole e 1	CN254402	0.0E+00 (2.45 ± 0.6)	3.9E-01 (1.25 ± 0.1)
	BIP0446	Unknown AgUNK5 [134]	CN254299	7.5E-03 (1.8 ± 0.5)	2.8E-03 (1.58 ± 0.3)
	BIP0504	Unknown AgUNK8 [243]	CN254350	2.2E-04 (2.03 ± 0.2)	6.5E-01 (1.05 ± 0.2)
	BIP1038	Unknown AgUNK9 [198]	CN254793	6.6E-03 (1.51 ± 0.3)	1.8E-02 (1.74 ± 0.8)
	BIP0462	Unknown [268]	CN254315	5.8E-02 (0.83 ± 0.1)	0.0E+00 (2.12 ± 0.2)
	BIP1242	Unknown [226]	CN254955	3.1E-03 (2.41 ± 2.5)	2.2E-04 (1.74 ± 0.8)
	BIP0352	Unknown	CN254216	8.1E-06 (1.42 ± 0)	2.4E-01 (2.46 ± 19.2)
	CTL082	Unknown	CB275397	3.4E-02 (1.3 ± 0)	1.7E-06 (2.38 ± 0.5)
	BIP0314	Unknown	CN254188	1.1E-03 (1.4 ± 0.2)	1.2E-04 (1.8 ± 0.7)
	BIP0697	Unknown	CN254514	2.2E-06 (3.04 ± 2.2)	3.6E-03 (1.37 ± 0.1)
	BIP1052	Unknown	CN254805	3.5E-05 (2.63 ± 1.6)	2.3E-01 (0.98 ± 0.3)
	BIP0483	Unknown	CN254335	3.8E-03 (1.97 ± 0.6)	2.0E-03 (1.31 ± 0.1)
	BIP0576	Unknown	CN254414	6.9E-05 (2.18 ± 0.3)	1.6E-02 (1.18 ± 0.1)
	BIP1182	Unknown	CN254906	2.2E-04 (1.84 ± 0.5)	7.3E-01 (1.12 ± 0.2)
	BIP1181	Unknown	CN254905	6.6E-02 (1.18 ± 0)	1.5E-03 (1.88 ± 0.7)

BIP0558	Unknown	CN254398	2.0E-02 (1.76 ± 1.1)	7.1E-03 (1.25 ± 0)
BIP0549	No score AgNS5 [189]	CN254389	1.1E-05 (1.96 ± 0)	8.1E-02 (1.28 ± 0.7)
BIP0272	No score AgNS4 [103]	CN254155	6.1E-03 (1.65 ± 0.2)	2.4E-03 (2.32 ± 1.7)
BIP1332	No score AgNS1 [118]	CN255026	5.0E-07 (2.19 ± 0.3)	3.3E-06 (1.41 ± 0)
BIP0317	No score [266]	CN254191	1.4E-02 (1.18 ± 0)	2.4E-04 (2.12 ± 0.6)
BIP0761	No score [260]	CN254567	7.1E-01 (1.09 ± 0.3)	7.6E-03 (2.38 ± 2.6)
BIP0455	No score [256]	CN254308	2.0E-03 (2.37 ± 1.5)	2.1E-02 (1.14 ± 0.2)
BRP041	No score [251]	CN255137	5.7E-03 (3.13 ± 9.8)	2.0E-03 (2.82 ± 4.6)
BIP0465	No score [209]	CN254318	1.2E-05 (2.55 ± 1.4)	5.0E-07 (1.7 ± 0)
BIP0530	No score	CN254372	9.1E-02 (2.51 ± 11.6)	8.5E-03 (3.19 ± 8.6)
BIP0997	No score	CN254757	5.5E-02 (1.88 ± 2.3)	2.1E-03 (1.75 ± 0.9)
BIP1183	No score	CN254907	4.6E-05 (1.6 ± 0.1)	1.5E-02 (2.32 ± 4.8)
BIP0303	No score	CN254181	1.8E-02 (0.92 ± 0.2)	8.3E-03 (2.18 ± 3.1)
BIP0246	No score	CN254136	2.1E-03 (1.37 ± 0.1)	8.8E-04 (2.1 ± 0.9)
BRP057	No score	CN255146	6.3E-01 (0.96 ± 0)	7.9E-03 (1.94 ± 1.8)
BIP0366	No score	CN254226	4.4E-02 (1.35 ± 0.1)	6.2E-03 (1.84 ± 1.1)
BIP0046	No score	CN253982	1.8E-02 (2.14 ± 1.8)	2.4E-04 (1.64 ± 0.4)
BIP0370	No score	CN254229	2.9E-05 (1.81 ± 0.3)	1.1E-03 (1.54 ± 0.3)
BIP1241	No score	CN254954	2.3E-06 (2.68 ± 0.3)	8.9E-04 (1.47 ± 0.1)
BIP1054	No score	CN254806	2.6E-06 (1.86 ± 0)	6.0E-04 (1.44 ± 0)
BIP1002	No score	CN254762	4.0E-03 (2.54 ± 4.3)	9.8E-03 (1.41 ± 0.2)
BIP0726	No score	CN254540	1.1E-02 (2.29 ± 5.3)	1.1E-02 (1.37 ± 0.3)
BIP0948	No score	CN254714	9.8E-03 (1.75 ± 0.9)	4.1E-02 (1.32 ± 0.3)
BIP0875	No score	CN254652	1.8E-04 (1.75 ± 0.1)	2.5E-03 (1.27 ± 0)
BIP1424	No score	CN255092	5.1E-04 (1.97 ± 0.5)	1.2E-02 (1.16 ± 0)
BIP0404	No score	CN254258	4.1E-03 (2.49 ± 2.1)	3.0E-02 (1.13 ± 0)

[]: contig group number (for redundant ESTs).

[*]: upregulation confirmed by Northern blotting.

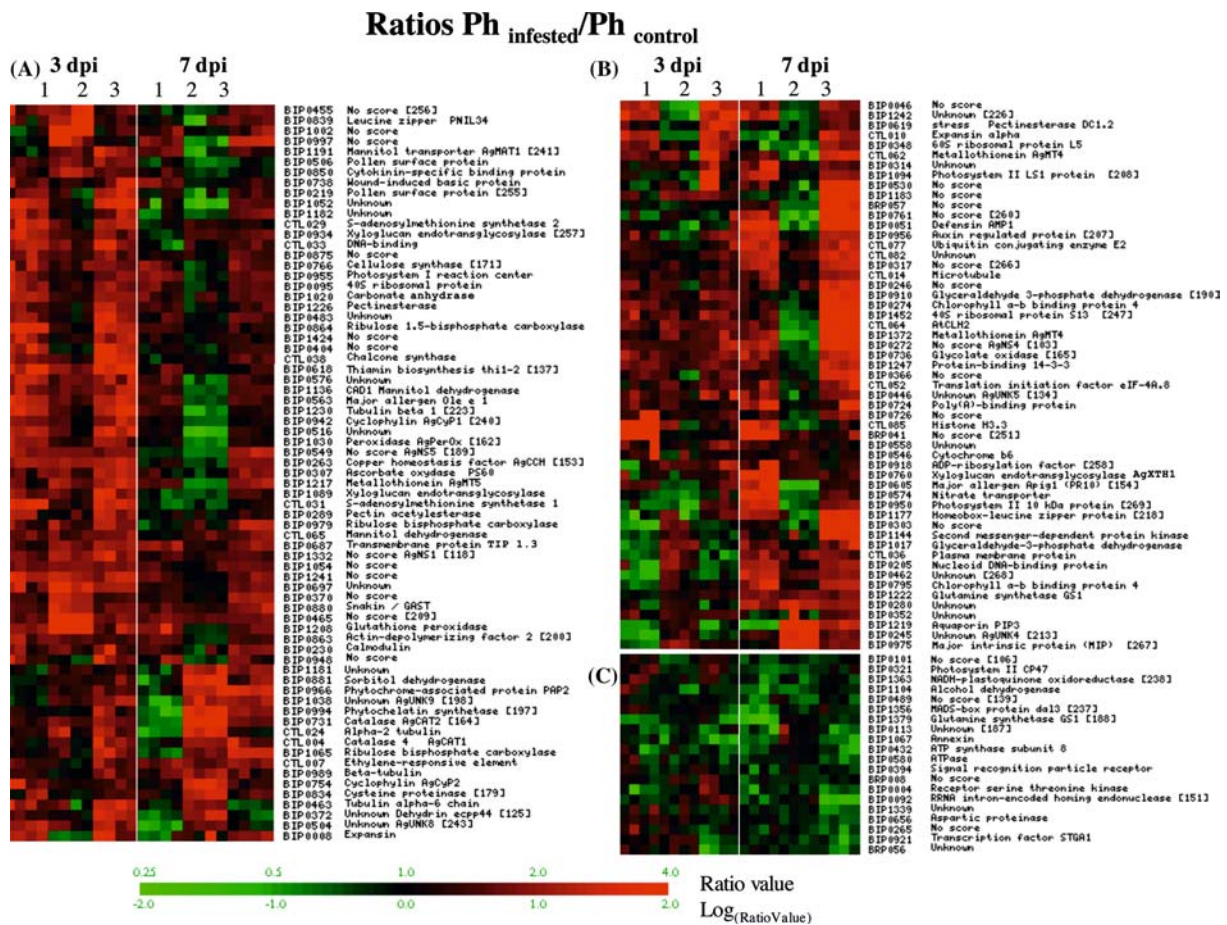


Figure 2. Clustering of the genes displaying the highest levels of up-regulation following aphid infestation. The 126 non-redundant genes selected on the basis of a $P_{\text{valCond}} < 0.01$ and an increase in expression by a factor > 1.7 were grouped in two clusters as described in (A) and (B), by hierarchical clustering. For comparisons, the subset of genes selected on the basis of a $P_{\text{valCond}} < 0.01$ that presented down-regulation are presented in (C). Each row corresponds to one clone spotted on the filter (i.e. one gene) and each column corresponds to the ratios calculated between the phloem of infested and non-infested plants (Ph: phloem) from normalised intensity values, for each experiment. Each clone was duplicated on the array and each spot was quantified independently on two sets of independent membranes. The three experimental replicates (numbered 1, 2, 3) therefore appear as successions of four columns for each time point. Ratios are shown in false colours as follows: Black corresponds to a ratio of 1, red indicates a ratio > 1 (up-regulated genes) and green indicates a ratio < 1 (down-regulated genes). The colour scale is indicated below the figure.

plants 3 or 7 dpi (Figure 2). The clusters showed a tendency also deduced from the distribution of average ratios made on the whole array (data not shown): the sequences displayed transient or stable up-regulation 3 or 7 dpi. The main class of genes (72 genes) displayed transitory up-regulation 3 dpi (Figure 2A), with expression levels returning to basal levels thereafter. Genes displaying up-regulation 7 dpi (55 genes) displayed a more variable response 3 dpi (Figure 2B), suggesting that these late-response genes also responded to other types of regulation. Twenty genes displayed up-regulation both 3 and 7 dpi.

The early-induced genes (3 dpi) included several genes encoding proteins involved in oxidative stress such as ascorbate oxidase, peroxidase, chalcone synthase, and enzymes linked to redox potential and metal homeostasis, such as a metallothionein MT5 and a copper chaperone factor (CCH). These genes included several encoding proteins with putative structural functions, such as a putative aquaporin (TIP1-3), two xyloglucan endotransglycosylases (XTH), two pectin methylesterases (PME), a pectin acetyl esterase and a cellulose synthase (CeS). Genes related to photosynthesis and carbon assimilation, such as those

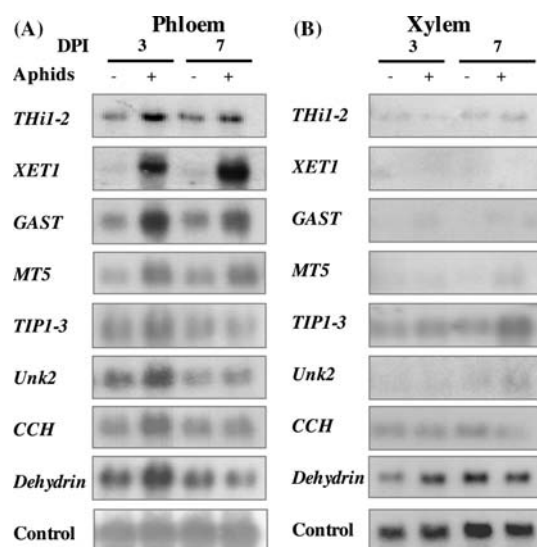


Figure 3. Differential expression of genes up-regulated in the phloem following infestation with aphids. Total RNA was extracted from phloem (Panel A) or xylem (Panel B) of non-infested and aphid-infested plants at two time points after infestation (3 and 7 days) as indicated. cDNA fragments encoding various phloem genes were used as probes to assess mRNA accumulation on northern blots of the corresponding genes. *THi1-2*: thiazole biosynthetic enzyme (clone BIP0467), *XTH1*: xyloglucan endotransglycosylase (clone BIP0760), *MT5*: metallothionein (clone BIP1217), *TIP1-3*: aquaporin (clone BIP0687), *GAST*: Snakin-like gibberellin-associated GAST (clone BIP0880), *Unk2*: unknown (clone BIP0280), *CCH*: copper chaperone (clone BIP0634), *Dehydrin* (clone BIP0372). We controlled for equal loading by probing for a catalase (clone SYD0317) which is stably expressed in the phloem and xylem of healthy and infested celery plants.

encoding the photosystem I reaction centre, Ru-BisCO, a chorophyllase, a carbon anhydrase, a thiazole biosynthesis enzyme (*Thi1-2*) and several chorophyll A/B binding proteins were also represented in this class, together with several genes encoding proteins of unknown function. The late-induced genes (7 dpi) included a different group of structural genes, encoding another xyloglucan endotransglycosylase (*XTH*), a plasma membrane protein, a second putative aquaporin (*PIP3*), and a melastatin-like protein. This set of genes included those encoding a protein associated with gibberellin-associated signalling (*Snakin2-GAST*), an ethylene-responsive element and the metallothionein *AgMT4*. It also included a number of genes encoding proteins of unknown function. Several genes involved in nitrate and sugar remobilisation were also found in this category, including glutamine synthase and mannitol dehydrogenase.

The up-regulation of several of these genes was confirmed by a Northern blot time-course analysis performed on a subset of sequences derived from the cDNA library and chosen to represent various functions (Figure 3). For most of these clones, the up-regulation observed on the Northern blot was more pronounced than that predicted from macroarray experiments. This confirms the validity of the approach used.

Tissue specificity of the aphid response

The tissue specificity of the changes in gene expression observed following aphid infestation was investigated by Northern blotting for the *GAST*, *Unk2*, *Thi1-2*, *XTH1*, *CCH*, *MT5*, *TIP1-3* and a dehydrin-like gene (Figure 3). Xylem tissues obtained from the petioles of the same infested and non-infested plants were used to determine whether these genes were equally induced in tissues other than phloem. In contrast to the significant induction observed in the phloem of infested plants, no clear induction was observed in the xylem 3 or 7 days after infestation for the *Thi1-2*, *XTH1*, *CCH* and dehydrin genes. In contrast, *TIP1-3* displayed moderate up-regulation 7 dpi, whereas this gene was induced 3 dpi in the phloem. Levels of mRNA accumulation were also lower in these tissues than in the phloem of non-infested plants (data not shown). This suggests that some of the genes identified as responding to aphid infestation were also more strongly expressed in vascular than in non-vascular tissues, despite the absence of screening for preferential vascular expression. Three of these genes, *CCH*, *MT5* and *Thi1-2*, displayed no detectable expression in parenchyma tissues, in healthy or infested plants (data not shown).

Stress specificity of the aphid response

We investigated the specificity of the response to aphids using the marker genes and plants challenged with another biotic stress, cucumber mosaic cucumovirus (CMV) infection. We used a second set of filters for hybridisation with probes prepared from the phloem of celery petioles from plants infected with cucumber mosaic virus, which causes systemic infections in celery. We carried out three experiments and the filters were treated as

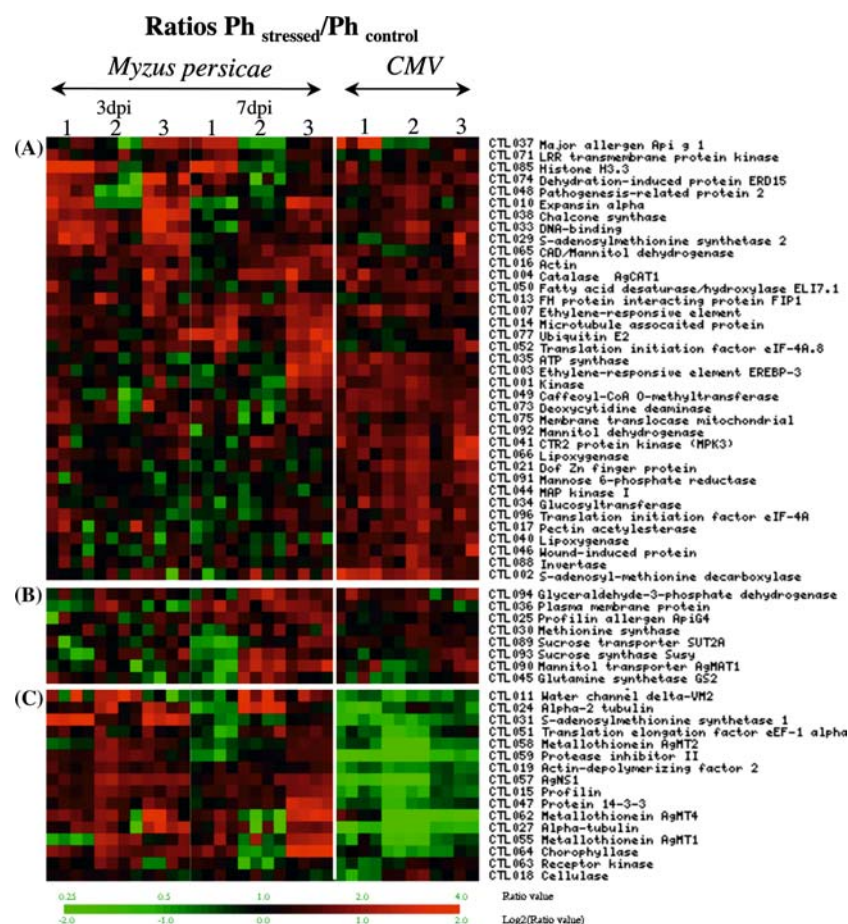


Figure 4. Divergent transcript profiling in the phloem of petioles for metabolic and stress marker genes following aphid infestation (*Myzus persicae*) and viral infection (cucumber mosaic virus). Hierarchical clustering of the expression patterns of control markers displaying differential expression according to biotic stress. Each row corresponds to one clone spotted on the filter (i.e. one gene) and each column corresponds to the ratios between the phloem of stressed and control plants, calculated from normalised intensity values for each experiment. Each clone was duplicated on the array and each spot was quantified independently on two set of membranes, appearing as successions of four columns. Aphid infestation experiments were carried out as described in Figure 2. We carried out three *Cucumber mosaic virus* (CMV) infection experiments, using strain I17F, (indicated 1, 2, 3 above the corresponding columns). Ratios are represented in false colours as follows: black corresponds to a ratio of 1, red indicates a ratio > 1 (up-regulated genes) and green indicates a ratio < 1 (down-regulated genes). The colour scale is indicated below the figure. (A) Subset of genes up-regulated by viral infection; (B) Subset of genes displaying only slight variations in response to aphid or virus stress; (C) Subset of genes down-regulated by viral infection.

previously described for quantification, normalisation and comparison. Expression profiles were clustered hierarchically (Figure 4). The response to aphids differed from the response to CMV. For example, the genes encoding metallothioneins, which were moderately (*AgMT1* and *AgMT2*) or strongly (*AgMT4*) induced by aphid infestation, were down-regulated in plants infected with CMV (Figure 4C). A similar pattern was observed for several genes encoding cytoskeleton-associated proteins, including one profilin, two α -tubulins and

an actin-depolymerising factor. In contrast, genes typically involved in plant defences, such as those encoding the PR2 protein, two lipoxigenases (LOX), a fatty acid desaturase (ELI7), a caffeoyl CoA methyltransferase and a chalcone synthase (CHS), showed more reproducible up-regulation in the phloem of plants infected by viruses than in the phloem of plants infested with aphids (Figure 4A). This suggests that the stress markers cloned in this study respond to pathogen infection and that the responses to aphids and plant viruses

are different. In contrast to the differential response to the two stresses observed for most markers, only moderate changes in the expression of genes associated with primary metabolism, such as *GS2*, *SUSY*, *MAT1* and *SUT2A*, were observed in response to both stresses (Figure 4B).

Materials and methods

Plant material and insect infestation, sampling and observation

Celery plants (*A. graveolens* var. Dulce cv. Vert d'Elné) were grown in a greenhouse in insect-proof cages under a 14-h-day cycle at 20 °C. They were infested at maturity (3–4 months old) between 11 a.m. and 1 p.m. Phloem strands were isolated from the petioles of immature and mature leaves as described by Daie (1987). Briefly, in detached petioles, vascular bundles, and then phloem and xylem strands can be separated from the parenchyma by manual peeling. We used this plant material to prepare cDNA libraries from purified phloem mRNA. We isolated xylem strands at the same time, from the same plants. The samples were frozen in liquid nitrogen directly after peeling.

Plant treatment: Myzus persicae infestation and virus infection

Green peach aphids are common on celery and inflict two types of damage: they stunt growth and may transmit viral diseases. The green peach aphid (*M. persicae*) strain SUS (Harpender) was acclimated on celery plants for 1 month before infestation of the plants analysed. We released aphids on intact celery plants to achieve infestation. Typically, three infested leaves from plants harbouring an acclimated population of aphids (> 100 aphids per leaflet) were applied to the upper surfaces of mature leaves on 3- to 4-month-old plants. Three days later, a few leaflets from the mature leaves of freshly infested plants harboured 10–20 adult aphids. Thereafter, infestation progressed rapidly, with large colonies (> 100 aphids, including nymphs and adults) present on most of the leaflets of mature leaves after 1 week. Control and infested plants were grown in separate insect-proof cages in the same growth chamber. We

infected plants with CMV (a tomato strain I17F), by the application of 10 µg of purified viral RNA per plant to leaves abraded mechanically with carborendum. Untreated control plants were grown in identical greenhouse conditions on independent tables and were harvested at the same time point. The petioles of developed leaves were harvested from control plants and from infected plants, after confirmation of successful infection by enzyme-linked immunosorbent assay (ELISA) 2–3 weeks after treatment.

Cloning of standard markers

The sequences of candidate genes were obtained from public databases. They were used to search for putative orthologous sequences in other plant species by similarity searches with BLASTN (Altschul *et al.*, 1997), using nucleotide databases (including ESTs) available at the NCBI [<http://www.ncbi.nlm.nih.gov/BLAST/>]. Sequences extracted from public nucleotide databases were aligned using the PileUp and ClustalW programs available in the GCG package (Genetics Computer Group, Wisconsin, USA). This made it possible to identify conserved regions, which we targeted using specific degenerate oligonucleotides. We carried out reverse transcriptase-polymerase chain reaction (RT-PCR) to generate cDNAs. The reaction mixture included 1 µg of RNA and we used standard reverse transcription and PCR conditions (94 °C for 2 min, then 30 cycles of 94 °C for 1 min, specific Tm for 30 s, and 72 °C for 1 min). The products were inserted into the pGEMTeasy vector (Clontech). The degenerate oligonucleotides used were, for LOX1: (+): 5'-AAAGATGAGCCWTGGTG GCC-3' and (-): 5'-GGGAAGAGCAARGTRTA TGG-3', CHS: (+): 5'-TTCATGATGTATCAG CAGGG-3' and (-): 5'-CAGAACAAGGAGTTC CAGTC-3'; EDR1: (+): 5'-TTGTTCGTTTTATG GGTGCT-3' and (-): 5'-ACTTCTGGTGCCAT CCACTC-3', PR10: (+): 5'-ATGTTGACACAG TCCTTCCC-3' and (-): 5'-TTAATTAGCTATGA GATAGG-3'; MPK4: (+): 5'-CCTCCGATCATG CCTATTGG-3' and (-): 5'-CAACCCACTGA CCAAAYATC-3'; HEL: (+): 5'-GTACTGCTCM ACKTGGGATG-3' and (-): 5'-TGCATTGRTC-CACKATTCTC-3'; 14-3-3 Protein: (+): 5'-GCCT ACAAGAACGTSATCGG-3' and (-): 5'-TGCA T GATGAGAGTGCTGTCYTTG-3'; PR2: (+): 5'-GGAGCAGGAACCATCAAGAAAG-3' and (-):

5'-TGGCAATCAGTAGGCACCAG-3'; Caffeoyl-CoA3-O-methyltransferase: (+): 5'-GAGAACCA GAGGCAATGAAAG-3' and (-): 5'-CACCAA CAGGAAGCATACAG-3'; ELI7.1: (+): 5'-AAA ATTCTCAACAACCCACCCG-3' and (-): 5'-CC CCTCAACCAATCCCATTC-3'; GS2:(-): 5'-GC ACAGTAATATGGMCCCTG-3' and (-): 5'-GCA CAGTAATATGGM CCCTG-3'; MAT1:(+): 5'-ATATTTTCAAGTGGCATGGG-3' and (-): 5'-C TACCTTGTGTCTCGGGGAA-3'. Additional clones were obtained from phloem cDNA libraries previously generated in the laboratory (Vilaine *et al.*, 2003) or provided by R. Lemoine (University of Poitiers – CNRS).

Construction of phloem cDNA SSH libraries

We isolated total RNA as described by Verwoerd *et al.* (1989). The cDNA SSH libraries were prepared from mRNA purified from the phloem of petioles of mature leaves sampled from non-infested plants and from infested plants 7 days after infestation. Subtracted double-stranded cDNAs were synthesised using the Smart-PCR cDNA Synthesis Kit (Clontech). The BIP library was obtained by subtractive hybridisation (SSH, Diatchenko *et al.*, 1996), using cDNAs derived from infested plants as the tester, and cDNAs from control plants as the driver, using the PCR-select cDNA Subtraction Kit according to the manufacturer's instructions (Clontech). The BIP library should therefore be enriched in genes expressed in infected plants and not expressed in control plants. The BRP library was obtained by subtractive hybridisation, with infested plant cDNAs used as the driver and control cDNAs used as the tester. The cDNA fragments were inserted into the pCRII-TOPO vector (Clontech).

Sequence analysis

Single-pass sequencing was carried out for randomly selected cDNA clones with the universal M13 reverse primer. The raw sequences were edited to mask vector, adaptor, poly(dA/dT), and inaccurate regions (> 0.2% of 'N'). Trimmed ESTs with valid sequences shorter than 80 nucleotides were eliminated. The quality of the sequences was confirmed by checking that no more than 0.1% of nucleotides; on average, were undetermined. Sequence analysis was performed with the GCG package (Genetics

Computer Group). The mean size of the edited ESTs was 255 bp, corresponding in most cases to the full-length SSH cDNA. Redundancy was assessed by iterative comparisons using BLASTN (Altschull *et al.*, 1997) performed against the EST library. Contigs were verified manually with 95% identity over > 80 bp used as the threshold for contig formation. Similarity searches were carried out with BLAST software (BLASTX, BLASTN, and TBLASTX), using databases available at the NCBI website [<http://www.ncbi.nlm.nih.gov/BLAST/>]. Supplementary analysis was performed at TAIR website [<http://www.arabidopsis.org/cgi-bin/BLAST>]. Sequences were classified into major functional categories essentially as in the MIPS functional catalogue [http://mips.gsf.de/cgi-bin/proj/thal/filter_funcat.pl]. Comparisons with standard phloem cDNA libraries were carried out using ESTs from the PA and PJ libraries derived from the phloem of adult and immature leaves, respectively, as previously described (Vilaine *et al.* 2003).

Preparation of cDNA macroarrays on nylon filters

Polymerase chain reaction products corresponding to the cDNAs were amplified from bacterial cultures using forward and reverse M13 universal primers (94 °C 2 min, then 30 cycles of 94 °C for 1 min, 55 °C for 30 s, 72 °C for 1 min and a final 15 min at 72 °C), and purified on Bio-Gel P60 gels (BioRad) by gel filtration at 350 × g. Purified PCR products were spotted at a mean concentration of 100 ng/μl on nylon membranes (8 × 12 cm) (NEN) and UV-linked in a UV Stratalinker 2400 apparatus (Stratagene). Spotting was performed using a Q-Bot robot (Genetix) equipped with a 384-well plotter. DNA products were arrayed in duplicate at a final density of nine spots per square, in a 3 × 3 grid pattern. The negative controls spotted on the filter included the PCR-amplified polylinker of pT-Adv (Clontech) and pCRII-TOPO (Clontech). Spotting was performed before sequencing of the clones; redundant clones were therefore spotted several times, providing additional internal controls for reproducibility.

Northern blot experiments, test and reference hybridisations

Northern blot analysis was performed with 10 μg of total RNA prepared as described by Verwoerd

et al. (1989). The RNAs were transferred onto a nylon membrane (Genescreen, Perkin Elmer Life Science) in $10 \times \text{SSC}$ (1.5 M NaCl, 0.15 M sodium citrate). Prehybridisation (4 h) and overnight hybridisation were performed at 65 °C, as described by Church and Gilbert (1984), and the membrane was then washed in moderately stringent conditions ($2 \times \text{SSC}$, 0.1% SDS at 65 °C, 2×15 min, followed by $1 \times \text{SSC}$ 0.1% SDS at 65 °C, 15 min and $0.1 \times \text{SSC}$, 0.1% SDS at 65 °C, 2×15 min). The cDNA probes, purified on Spin-X columns (Costar), were labelled with the Prime-a-gene Labelling kit (Promega) in the presence of $\alpha^{32}\text{P}$ dCTP. Control loading and transfer were assessed by ethidium bromide staining.

Macroarray test hybridisations were carried out with complex probes prepared from total RNAs extracted from individual plants. Probes were prepared by reverse transcription in the presence of $\alpha^{32}\text{P}$ dCTP and 50 µg of total RNA, as described by Vilaine *et al.* (2003). Prehybridisation (4 h) and overnight hybridisation were performed at 42 °C, in Church and Gilbert's buffer supplemented with 50% formamide. Washing conditions were identical to those used for Northern blotting. The filters were placed against imaging plates for 16 h and signals were detected with a bioimager analyser (Fuji Bas1500). A maximum of three successive hybridisations, including the T7 reference hybridisation, were carried out for each filter. The membrane was stripped by incubation with 0.4 N NaOH, 0.1% SDS (w/v) at 65 °C for 2×15 min between probes. We checked that there was no residual radioactivity after stripping.

Reference hybridisations were carried out using 100 ng of the T7 primer (20-mer) labelled with the T4 polynucleotide kinase (InVitrogen), as recommended by the manufacturer, in the presence of 50 µCi of $\gamma^{32}\text{P}$ -ATP. Prehybridisation and hybridisation were performed at 42 °C in the buffer described by Church and Gilbert (1984), without formamide, and membranes were then washed ($2 \times \text{SSC}$, 0.1% SDS at 42 °C, 2×10 min, then $1 \times \text{SSC}$, 0.1% SDS at 42 °C, 10 min, and $0.1 \times \text{SSC}$, 0.1% SDS at room temperature, 10 min). Reference hybridisations (T7) were used to select membranes with homogeneous spotting, as determined by the correlation coefficient for signals emanating from the same dot. Only sets of membranes with high correlation coefficients (> 0.98) were used for further test hybridisations. This step

ruled out filters displaying irregular spotting. Clones with spotting abnormalities (including duplication irregularities or low concentrations, i.e. an intensity value less than twice the background) were identified using reference hybridisation.

Macroarray data analysis

Signal intensities were quantified with a Xdots-Reader (Cose-Paris). A predefined grid, determining the area of signal quantification, was manually set up for recording. The signal for each dot was quantified by summing the intensities of all pixels within a circle of fixed radius after subtracting the local background. The blots were normalised as previously described (Vilaine *et al.*, 2003) except that we did not perform the initial normalisation step using the mean intensity calculated for the whole set of dots. This step was omitted to limit bias due to the use of subtracted libraries.

Briefly, a subset of 50 clones with the smallest variation coefficients (standard deviation/mean of the intensities) was determined by stepwise calculation for the entire set of hybridisation data. The accuracy of the method was confirmed by calculating the mean ratio for the condition comparisons (S/T ratios), which was close to 1 (± 0.37). Hybridisation and sequence data were analysed and stored in a database implemented with 4D (ACI, Paris).

Statistical analysis and clustering

We used ANOVA applied to normalised intensity values, using four parameters: clone, condition, experiment (i.e. set of plants), and clone duplication. Local ANOVA made it possible to associate a $P_{\text{val}_{\text{cond}}}$ with each clone spotted on the filters. We used a threshold of significance of 0.01 for this P_{val} , as frequently used in other studies. We focused on differences in expression level between conditions of potential biological relevance, by implementing a 70% increase threshold (S/T ratio > 1.7). This threshold was applied to the mean ratio for the normalised intensity values calculated for the comparisons (S/T ratios) and determined in independent experiments. This ratio gave an indication of the magnitude of variations in the various conditions. Cluster analysis was performed

on ratios calculated from the normalised intensity values after hybridisation with probes corresponding to the two conditions considered (infested/non-infested) using XCluster (Sherlock, 2000 [<http://genome-www.stanford.edu/~sherlock/cluster.html>]). ANOVA and cluster analysis were performed via the "Interfiltres" interface (Vilaine *et al.*, 2003).

Discussion

When a plant is attacked by a pathogen or an insect, it displays a response at the whole-organism level involving both primary and systemic (or distal) responses. Both are initiated at the cellular level by signal transduction cascades, followed by the fine-tuning readjustment of gene expression involved in stress resistance (Taylor *et al.*, 2004). Responses are then relayed by intercellular events leading to the production of local and long-distance signal molecules that trigger further changes in gene expression in cells not directly injured. Many molecular studies have specifically focused on the local and systemic responses to injury, but most have failed to reveal the precise spatio-temporal sequence of events in signalling pathways. Genetic studies using mutants altered in individual components of the pathways are a powerful tool for deciphering the chronology of events leading to protection against injury. Alternatively, events may be uncoupled by looking at the responses taking place in different tissues or at different time points. Such approaches should provide a useful framework for mechanistic models.

Systemic phloem response to aphid feeding

Celery (*A. graveolens*) is one of the hosts of the green peach aphid (*M. persicae*), a generalist phytophagous insect that produces multiple generations on mature leaf blades. Celery petioles, which remain insect-free, are an ideal material for separating phloem from the surrounding tissues and for following differential gene expression (Vilaine *et al.*, 2003). Moran and Thompson (2001) showed that the systemic response to *M. persicae* infestation is initiated after 3–4 days in *Arabidopsis* plants. We therefore analysed the systemic phloem response in celery plants infested

by *M. persicae*, 3 and 7 dpi, confining our study to the petioles, which are not directly wounded by the aphids. We identified 891 non-redundant genes expressed at various levels in the phloem of healthy and infested plants and used them to construct a set of cDNA macroarrays, which we then hybridised with probes corresponding to the phloem of healthy or infested plants. The transcript profile, deduced from the cDNA macroarrays, was then analysed by ANOVA (Kerr *et al.*, 2000, Draghici *et al.*, 2003) to identify groups of genes differentially expressed in response to aphid infestation.

A large number of genes involved in diverse functions were induced following aphid attack, paralleling the responses observed in organs in other plant–aphid systems (Moran *et al.*, 2002; Zhu-Salman *et al.*, 2004). The genes induced included those encoding proteins involved in primary and secondary metabolism, photosynthesis and housekeeping functions, consistent with reports for other plant–insect interactions (Taylor *et al.*, 2004). The expression of housekeeping genes is not specific for particular tissues or cell layers and the induction of these genes in response to aphids probably reflects a general stress response rather than a specific phloem response. Several genes encoding proteins frequently associated with signal transduction, the expression of which may be restricted to specific cell types, were up-regulated in celery phloem following aphid attack. The genes concerned encoded a calmodulin, a 14-3-3 binding protein, a kinase and several DNA binding factors. As little is known about the mechanisms of signal transduction in the phloem, further studies on stress and tissue specificity are required before definitive conclusions can be drawn. However, these findings illustrate the potential of such approaches for targeting new specific signalling pathways in the phloem, which is difficult when evaluating responses at the whole-organ level. They also demonstrate the complementary nature of this approach with respect to the most widely used models, such as *Arabidopsis* and sorghum (Moran *et al.*, 2002; Zhu-Salzman *et al.*, 2004).

The systemic response in the phloem encompasses the general oxidative stress response

Several stress-related genes associated with oxidative stress – encoding three catalases, a peroxi-

dase, a glutathione peroxidase and an ascorbate oxidase – displayed significant up-regulation. This is consistent with injury due to aphid feeding inducing the production of reactive oxygen species (ROS) at long distance in the phloem, as hypothesised in several biochemical and molecular studies (Argandona *et al.*, 2001; Ni *et al.*, 2001; Moran *et al.*, 2002; Zhu-Salman *et al.*, 2004). We also observed the up-regulation of genes encoding metal ion scavengers such as metallothioneins displaying preferential expression in the vascular tissues of several species, including celery (Mira *et al.*, 2001; Guo *et al.*, 2003; Vilaine *et al.*, 2003). The gene encoding the CCH copper chaperone, which has been reported to act as an antioxidant (Mira *et al.*, 2001; Akashi *et al.*, 2004; Mir *et al.*, 2004), also seemed to be specifically induced in the phloem after aphid infestation (Figure 3), supporting the hypothesis of a phloem-specific response. The differential expression of such genes in the phloem is particularly interesting because the internal oxygen content of this tissue is low (Van Dongen *et al.*, 2003), and apoxia affects redox state and the formation of ROS (Blokina *et al.*, 2003). This suggests that even the more classical responses to stress may have specific counterparts in the phloem, and our findings provide evidence for the tissue-specific control of oxidative stress in response to aphid infestation.

Antimicrobial peptides and small proteins

Several genes annotated in databases as stress-responsive or stress-related were found to be induced in response to aphid attack. They encoded the major celery allergen ApiG1/PR10-like (Breiteneder *et al.*, 1995); a cytokinin-binding protein similar to proteins of the PR10 family (Park *et al.*, 2004); a putative snakin2-GAST, which acts as a stress-induced antimicrobial peptide (Berrocal-Lobo *et al.*, 2002); a dehydrin phosphoprotein, ecpp44 (Allagulova *et al.*, 2003), and a defensin (Thomma *et al.*, 2002). Genes encoding putative small antimicrobial proteins have been reported to be induced in responses to various stress (Veronese *et al.*, 2003). The precise function of these proteins is unknown, but they are thought to be ubiquitous. Other ubiquitous factors, such as the chaperones HSP70 and HSP90, play key roles in buffering physiological and developmental variations (Sung and Guy, 2003;

Bergman and Siegal, 2003). These small proteins may therefore act at multiple levels to maintain homeostasis and or protein stability during stress. Genes encoding several such proteins, from various gene families, were shown to be preferentially expressed in vascular tissues in some species. For example, *Medicago sativa* PR10 is up-regulated in the vascular tissues in response to *Pseudomonas syringae* attack (Breda *et al.*, 1996). Several dehydrins display vascular expression in tomato and *Arabidopsis* (Godoy *et al.*, 1994; Nylander *et al.*, 2001). Defensin genes related to the one identified in this study were also found to be expressed in the vascular tissues of pea (Almeida *et al.*, 2000). It is currently unclear whether the response in celery phloem differs from that in other tissues. The production of small putative antimicrobial proteins may be one of the specific mechanisms of the basic phloem response. The preferential expression of many stress-related sequences in celery phloem is consistent with this view (Vilaine *et al.*, 2003), and the phloem sap of cucurbits is known to contain a large number of defence-related proteins (Walz *et al.*, 2004). Furthermore, the *Snakin2-GAST* gene was induced specifically in the phloem, and not in the xylem, following aphid infestation (Figure 3).

Involvement of defence mechanisms in the phloem

We also analysed the response of putative stress markers. Several stress-related markers identified in other species (Petersen *et al.*, 2000; Frye *et al.*, 2001; Veronese *et al.*, 2003; Table 2 for more details) were also studied and their transcript profiles analysed. Most markers displayed less marked differential expression than observed in the response to viral infection (Figure 4). Consistent with this finding, genes encoding proteins directly involved in defence mechanisms, such as PR proteins and enzymes involved in the biosynthesis of SA, JA or ethylene, were underrepresented in the ESTs derived from the subtracted phloem cDNA libraries. Voelckel *et al.* (2004) also reported that aphids elicited only weak responses from stress-related genes in the local and systemic responses of *Nicotiana attenuata* plants. However, other studies have reported the induction by aphids of several SA- and JA-related genes, such as *PR1*, *BGL2*, *LOX1*, *LOX2*, and of protease inhibitor genes (Moran and Thompson, 2001;

Martinez de Ilarduya *et al.*, 2003; Zhu-Salzman *et al.*, 2004).

There is no obvious proof that these genes should be up-regulated systemically in the phloem and, indeed, *LOXD* and *PR2* are not induced systemically in *Arabidopsis* or tomato. The phylogenetic divergence between Apiaceae, Solanaceae and Cruciferae may also result in differences in the transcriptional regulation of such genes. Furthermore, the up-regulation of gene expression in the phloem in response to stress has been little studied and the control of expression may differ between the phloem and other tissues.

Modifications to primary and secondary metabolism

The phloem is the principal route used for the long-distance translocation of photoassimilates, under the control of various carriers and transporters, dependent upon carbon and nitrogen metabolism (Sjölund, 1997; Oparka and Turgeon, 1999; Van Bel, 2003) and regulated by a number of stimuli, including stress (Roitsch, 1999). Several ESTs identified in this study were related to genes encoding components of carbon and nitrate assimilation and partitioning. However, the increase in expression of such genes was only moderate in celery plants infested with *M. persicae*. For example, the transcript profile of three glutamine synthetase (*GS*) genes (two *GS1* and one *GS2*) was studied and only the *GS1-1* gene was slightly up-regulated. The genes encoded by these enzymes are involved in nitrogen remobilisation in the phloem during events such as senescence leading to the release of free amino acids in the translocation stream. The induction of such genes is consistent with the changes in amino acid content observed in response to aphid feeding (Sandström *et al.*, 2000; Gironse *et al.*, 2003). Voelckel *et al.* (2004) also reported the induction of glutamate synthase (Fd-GOGAT) in *N. attenuata* infested with *M. nicotianae*. However, our study provides only weak evidence for transcriptional effects on nitrogen remobilisation in the phloem of infested leaves because only moderate induction was observed. Two genes associated with polyol sugar metabolism and partitioning, encoding mannitol dehydrogenase and the mannitol transporter, were more strongly up-regulated. The petioles of celery var. dulce store mannitol and the up-regulation of these two genes may reflect polyol sugar remobil-

isation in response to the decrease in carbohydrate supply from source leaves colonised by aphids. Mannitol also acts as an osmoprotectant in celery (Everard *et al.*, 1994), and the differential expression of these two genes may also reflect an osmotic stress response.

In contrast, genes encoding several carbon assimilation enzymes (carbonic anhydrase, glycolate oxidase, ascorbate oxidase, glyceraldehyde-3-phosphate dehydrogenase and S-adenosyl methionine synthetase) were strongly induced 3 dpi. This effect was attenuated 7 dpi. This pattern of differential expression parallels that for genes involved in photosynthesis (encoding chloroplast components, photosystem I, chlorophyll a-b binding protein, phytochrome, chlorophyllase and RuBisCo), which are slightly up-regulated shortly after infestation (3 dpi). Changes in photosynthetic activity have been reported in some plant-aphid interactions (Macedo *et al.*, 2003), but such responses are generally associated with the physiological status of the plant. No change in photosynthetic activity was observed in the leaves of infested celery (Divol, unpublished results). The implications of these differential expression patterns are unclear, as they may reflect indirect effects on several pathways.

Thiamine biosynthesis

A gene encoding an enzyme involved in thiamine biosynthesis, *Thi1-2*, was strongly and specifically induced in the phloem, as confirmed by Northern blotting (Figure 3). Thiamine is an essential cofactor required for the activity of several enzymes associated with key metabolic pathways including the Krebs's cycle, glycolysis, the pentose phosphate cycle and pigment biosynthesis (Belanger *et al.*, 1995). It is also thought to induce PR proteins and to be involved in defence responses (Malamy *et al.*, 1996). We previously showed that at least one other *Thi1*-like gene was transcribed preferentially in the vascular tissues (Vilaine *et al.*, 2003). These two genes seem to be regulated differently by aphid feeding. Further analysis is required to determine the contribution of each gene to the stress response. Thiamine is an important vitamin (B1), and aphids are essentially auxotrophic for most vitamins, although aphid-associated symbiotic bacteria supply many metabolites not provided in the phloem sap. The

release of thiamine into the phloem may therefore be beneficial for the aphids. However, the role played by thiamine as a cofactor in many key metabolic reactions suggests it may be involved in the regulation of a yet unidentified biosynthesis pathway.

Cell wall modifications and plasma membrane permeability

Several genes involved in cell wall modifications appeared to be up-regulated in infested plants. They included genes encoding enzymes involved in the modification of pectins (PME and PE) and hemicelluloses (XTH) and in the synthesis and expansion of the cellulose matrix (cellulose synthase and expansin). Cell wall modifications are frequently induced in response to pathogens (Schenk *et al.*, 2003; Lee *et al.*, 2004), phytophagous insects (Hui *et al.*, 2003; Voelckel and Baldwin, 2003) and nematodes (Goellner *et al.*, 2001; Vercauteren *et al.*, 2002) and are usually associated with the strengthening of extracellular barriers (Esquerre-Tugaye *et al.*, 2000) or the release of signalling molecules (Vorwerk *et al.*, 2004). Cell wall modifications have not been extensively described in response to aphids, but some molecular evidence has now been obtained to suggest that such modifications occur. Moran *et al.* (2002) reported the induction of a *TCH4* xyloglucan endotransglycosylase gene (*XTH22*) in *Arabidopsis* following aphid attacks. Voelckel *et al.* (2004) recently demonstrated the local induction of a *XTH* in *N. attenuata* infested with *M. nicotianae*. It is unclear whether the induction of such genes in the phloem reflects general defence mechanisms or more specific events. Callose deposition in phloem sieve elements has been observed in response to various aphids (reviewed in Moran *et al.*, 2002), but the role of callose in the injury response remains unclear (Van Bel *et al.*, 2002). The various cell wall-associated genes up-regulated in celery in response to aphid infestation have been reported to be involved in cell wall expansion during plant growth, development and adaptation in other species (Wu *et al.*, 2001; Darley *et al.*, 2001). The significant increase in *XTH1* mRNA levels was confirmed by Northern blotting. This increase seemed to be limited to the phloem, as no activity was observed in the xylem. This observation strongly supports the

hypothesis of the setting up of specific phloem responses to aphid infestation.

Cell wall expansion depends on turgor pressure (Cosgrove, 1999), which is associated with osmotic potential and the transport coefficient for water uptake. The permeability of the membrane to water is controlled by plasma membrane water channels such as aquaporins and putative plasma membrane intrinsic proteins (MIP). During prolonged aphid feeding, both osmotic potential and water uptake may be affected because the genes involved in mannitol allocation and those encoding aquaporin and MIP may be differentially expressed. Thus, changes in cell wall properties and turgor pressure may counterbalance possible changes in source-sink relationships resulting from the removal of significant volumes of phloem sap during aphid infestation (Girousse *et al.*, 2003). The main driving force for the transport of phloem sap from source to sink organs (mass flow) is the osmotically generated hydrostatic pressure between source and sink organs (Van Bel, 2003), as initially described by Münch (1930). This hydrostatic pressure is affected by variations in turgor pressure and osmotic pressure in the source or transport organs. Models have been developed to describe phloem water transport (Pritchard *et al.*, 2000; Daudet *et al.*, 2002), but these models are insufficiently detailed as concerns local water entry and the expression of various phloem-specific aquaporins (Schäffner *et al.*, 1998; Barrieu *et al.*, 1998; Kirch *et al.*, 2000; Frangne *et al.*, 2001) affecting the resistance of membranes to water (turgor), is not integrated into these models. Aphid feeding affected regulation of the transcription of genes involved in cell wall modification and water entry in the phloem highlights the possibility that there may be specific regulatory pathways in the phloem for the local control of turgor pressure. We need to determine what modifications actually take place in sieve elements, particularly as concerns cell water properties, osmotic gradient and turgor pressure in response to aphid infestation.

The molecular response to aphid feeding in celery phloem parallels certain aspects of interactions described in *Arabidopsis*, *N. attenuata*, sorghum and tomato in terms of general stress gene expression (Moran and Thompson, 2001; Moran *et al.*, 2002; Martinez de Iladuya *et al.*, 2003; Zhu-Salzman *et al.*, 2004; Voelckel *et al.*, 2004). Other

responses, such as the differential expression of genes encoding an enzyme involved in vitamin B1 biosynthesis, several cell wall-modifying enzymes and water channels, were not reported in other systems. It remains unclear whether these differences reflect a species-specific effect or whether they are simply masked in studies assessing whole-organ responses. However, this study indicates that the phloem *per se* is actively involved in multiple modifications and suggests that the response to aphid attack involves at least some tissue-specific mechanisms.

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