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Active defence responses associated with non-host resistance of *Arabidopsis thaliana* to the oomycete pathogen *Phytophthora infestans*

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SUMMARY

The molecular basis of non-host resistance, or species-specific resistance, remains one of the major unknowns in the study of plant–microbe interactions. In this paper, we describe the characterization of a non-host pathosystem involving the model plant *Arabidopsis thaliana* and the economically important and destructive oomycete pathogen *Phytophthora infestans*. Cytological investigations into the early stages of this interaction revealed the germination of *P. infestans* cysts on *Arabidopsis* leaves, direct penetration of epidermal cells, formation of infection vesicles and occasionally secondary hyphae, followed by a typical hypersensitive response. *P. infestans* biomass dynamics during infection of *Arabidopsis* was monitored using kinetic PCR, revealing an increase in biomass during the first 24 h after inoculation, followed by a decrease in the later stages. Transgenic reporter lines and RNA blot analyses were used to characterize the defence responses induced following *P. infestans* infection. Significant induction of *PDF1.2* was observed at 48 h after inoculation, whereas elevated levels of *PR* gene expression were detected three days after inoculation. To further characterize this defence response, DNA microarray analyses were carried out to determine the expression profiles for *c.* 11 000 *Arabidopsis* cDNAs 16 h after infection. These analyses revealed a significant overlap between *Arabidopsis* non-host response and other defence-related treatments described in the literature. In particular, non-host response to *P. infestans* was clearly associated with activation of the jasmonate pathway. The described *Arabidopsis*–*P. infestans* pathosystem offers excellent prospects for improving our understanding of non-host resistance.

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INTRODUCTION

Plants are challenged by numerous pathogens throughout their life cycles and yet are able to fend off most infections. Indeed, in interactions between plants and microbial pathogens, resistance is the rule and disease the exception. This phenomenon is known as non-host resistance or species-specific resistance and is thought to explain why a pathogen can cause disease in particular plant species but not in others. Understanding the molecular basis of non-host resistance remains one of the elusive quests in the study of plant–microbe interactions. Pre-formed barriers and compounds such as saponins are ubiquitous in plants and play an important role in non-host resistance to filamentous fungi (Morrissey and Osbourn, 1999; Osbourn, 1996). However, most contemporary models of non-host resistance evoke a complex overlay of specific resistance and nonspecific defence responses (Gomez-Gomez and Boller, 2002; Heath, 2000; Kamoun *et al.*, 1999; Kamoun, 2001; Nurnberger and Brunner, 2002). Specific resistance has been extensively studied in host pathosystems and typically follows Flor's gene-for-gene model. In this model, resistance is determined by the simultaneous expression of a pathogen avirulence (*Avr*) gene with the corresponding plant resistance (*R*) gene, leading to the hypersensitive response (HR), a general defence response of plants that includes apoptotic cell death (Dangl and Jones, 1998; Flor, 1971; Staskawicz *et al.*, 1995). The extent to which the gene-for-gene model can be expanded to non-host interactions remains unclear. However, we and others have speculated that in many pathosystems non-host resistance can be explained by the occurrence of an arsenal of *R* genes that recognize multiple or essential *Avr* genes (Heath, 2000; Kamoun *et al.*, 1998; Kamoun *et al.*, 1999; Kamoun, 2001; Staskawicz *et al.*, 1995).

The oomycetes represent a diverse and phylogenetically unique branch of eukaryotic microbes that includes many important pathogens of plants (Baldauf *et al.*, 2000; Margulis and Schwartz, 2000; Sogin and Silberman, 1998). The most notorious

oomycetes are *Phytophthora* species, arguably the most devastating pathogens of dicot plants (Erwin and Ribeiro, 1996; Kamoun, 2000; Kamoun, 2003). For example, *Phytophthora infestans* causes late blight, a devastating disease that results in multibillion-dollar losses in potato and tomato production (Fry and Goodwin, 1997a,b; Garelik, 2002; Smart and Fry, 2001). Most plants, such as weeds and various crops, are resistant to *P. infestans*, and grow unimpaired in or next to fields with a high incidence of late blight (Colon *et al.*, 1992; Kamoun *et al.*, 1999). Understanding the molecular basis of non-host resistance to *P. infestans* will provide insight into a key molecular process and will offer novel perspectives for engineering durable late blight resistance in crop plants.

A first insight into the basis of non-host resistance to *Phytophthora infestans* came through detailed cytological analyses. Microscope observations revealed penetration of epidermal cells by *P. infestans* in all examined interactions, including those with plant species unrelated to the solanaceous hosts (Gross *et al.*, 1993; Kamoun *et al.*, 1998; Kamoun *et al.*, 1999; Naton *et al.*, 1996; Schmelzer *et al.*, 1995; Vleeshouwers *et al.*, 2000). Fully resistant plants, such as the non-hosts *Solanum nigrum*, parsley and tobacco display a typical localized HR at all infection sites (Colon *et al.*, 1992; Kamoun *et al.*, 1998, 1999; Naton *et al.*, 1996; Schmelzer *et al.*, 1995; Vleeshouwers *et al.*, 2000). The HR can be highly localized to a single epidermal cell or can affect a group of cells surrounding the penetrating hyphae, depending on the interaction examined (Kamoun *et al.*, 1998; Vleeshouwers *et al.*, 2000). The view that has emerged from these studies is that the HR, perhaps mediated by *R* genes, is associated with all known forms of genetic resistance to *P. infestans* including non-host resistance (Kamoun *et al.*, 1999; Kamoun, 2001).

Some of the *Phytophthora* molecules that trigger the HR or other defence responses in non-host plants are known. Species-specific elicitors have been described in *P. infestans* and other *Phytophthora* species and can trigger defence responses in non-host plants. For example, an extracellular transglutaminase that is conserved in *P. infestans* and other *Phytophthora* species induces defence responses in the non-host parsley (Brunner *et al.*, 2002; Nurnberger and Brunner, 2002). Members of the INF elicitin family induce the HR and related biochemical changes specifically in *Nicotiana* (Kamoun *et al.*, 1997, 1998; Sasabe *et al.*, 2000). *P. infestans* strains deficient in the elicitin INF1 induce disease lesions on *Nicotiana benthamiana*, suggesting that INF1 functions as an *Avr* factor that conditions resistance in this species (Kamoun *et al.*, 1998). Using gene silencing, Peart *et al.* (2002) recently showed that the response of *N. benthamiana* to INF1 was dependent on the ubiquitin ligase-associated protein SGT1, which is also required for non-host resistance to bacterial plant pathogens. The *N. benthamiana* pathosystem holds great promise for dissecting elicitor response and resistance to *P. infestans*, since this plant is amenable to high-throughput functional assays using virus-induced gene silencing (VIGS) (Baulcombe, 1999). Nevertheless, the *N. benthamiana*–*P. infestans* interaction does not qualify as a strict non-host pathosystem since some wild-type isolates of *P. infestans* were recently found to infect this plant (F. Govers, personal communication; C. Smart & W.E. Fry, personal communication) (Kamoun, 2001).

We elected to employ *Arabidopsis thaliana* as a model for understanding non-host resistance to oomycete pathogens. Several biotrophic oomycetes, such as *Peronospora parasitica* and *Albugo candida*, are known to infect *Arabidopsis* (Holub *et al.*, 1995; Parker *et al.*, 1996; Rehmany *et al.*, 2000; Reignault *et al.*, 1996). Cabbage isolates of *Phytophthora brassicae* (previously known as *Phytophthora porri*) (Roetschi *et al.*, 2001) and several isolates of *Phytophthora cinnamomi* (Robinson & Cahill, 2003) can also infect *Arabidopsis*, and these pathosystems are expected to facilitate the study of host infection by *Phytophthora*. However, most *Phytophthora* species, such as *P. infestans*, and the root pathogen *Phytophthora sojae* cannot infect *Arabidopsis* suggesting that this plant forms an untapped source of resistance to *Phytophthora* (Kamoun *et al.*, 1999; Kamoun, 2001; Takemoto *et al.*, 2003). Considering the impressive set of functional genomic resources that are available, *Arabidopsis* offers good prospects for dissecting the complex interactions that take place between a non-host plant and an oomycete pathogen and forms both an alternative and a complementary system to ongoing work on the resistance of *Nicotiana* to *P. infestans*. In this study, we describe the characterization of a non-host pathosystem involving *Arabidopsis* and an economically important *Phytophthora* species. Using cytological and molecular analyses, as well as microarray gene expression profiling, we obtained an overview of the active defence responses associated with the non-host resistance of *A. thaliana* to *P. infestans*.

RESULTS

Interaction between *Arabidopsis* and *P. infestans*

To characterize the interaction between *Arabidopsis* and *P. infestans*, we performed repeated inoculations of *Arabidopsis* with *P. infestans* zoospores. We tested numerous inoculation parameters, including *Arabidopsis* leaves at the seedling or rosette stage, multiple combinations of *Arabidopsis* ecotypes and *P. infestans* strains, detached vs. attached leaves, and drop vs. spray inoculations. In all treatments, late blight lesions and sporulation were never observed, whereas infection of the host plant tomato was observed under most of the conditions tested. Normally, no macroscopic symptoms could be detected on *Arabidopsis*, but occasionally, discrete necrotic specks typical of the HR could be observed at the inoculation site, particularly when highly concentrated zoospore solutions were used.

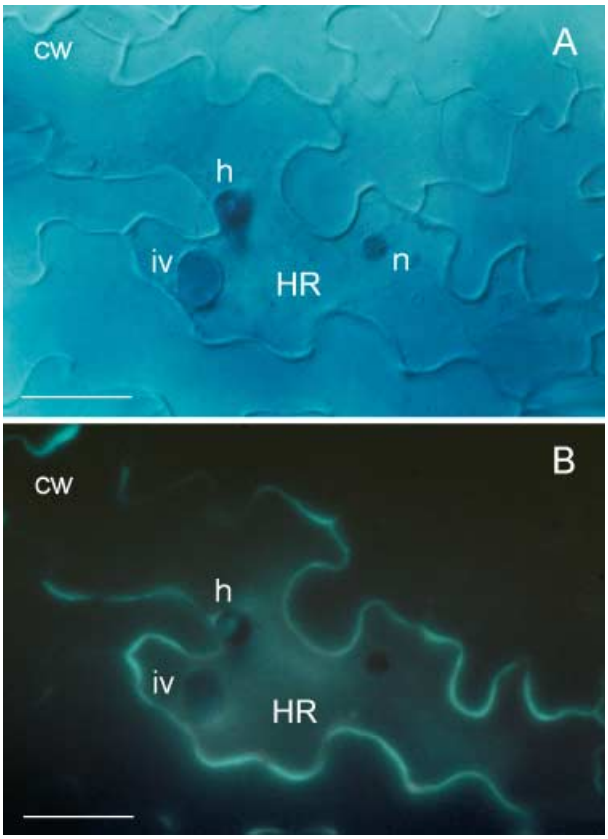

Fig. 1 Hypersensitive response (HR) in *Arabidopsis*, 46 h after inoculation with *Phytophthora infestans*. Upon penetration of *P. infestans*, an infection vesicle and a secondary hypha were formed, and the HR was induced in a single epidermal cell. Characteristics of the HR cell are: (A) granular structure of the cytoplasm noted with DIC optics, (B) fluorescence of cytoplasm and cell wall with UV illumination and thickening of the cell wall. cw, thickened cell wall; h, hyphae; HR, HR cell; iv, infection vesicle; n, nucleus, bar = 15 μm.

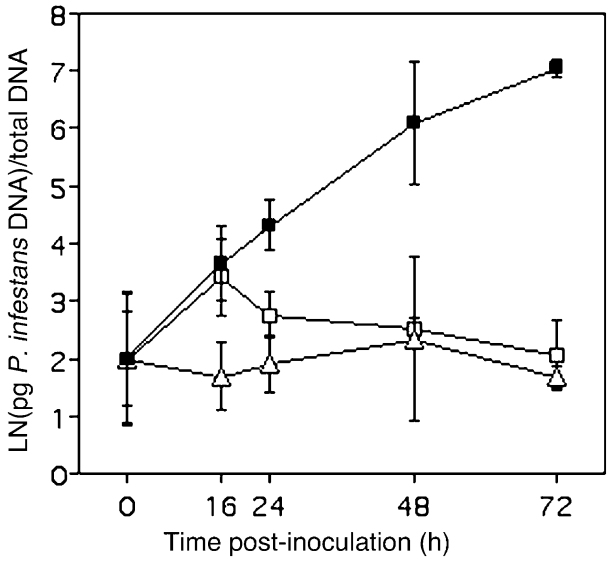

Fig. 2 Kinetic PCR quantification of *Phytophthora infestans* biomass upon germination and penetration of *Arabidopsis* (non-host, open squares), tomato (host, solid squares) and in the absence of a plant substrate (no-host, open triangles). Zoospore suspensions of *P. infestans* were used to inoculate the respective plants. Four samples, each containing four leaf discs, were harvested at 0, 16, 24, 48 and 72 h after inoculation and used for DNA extraction. Four uninoculated leaf discs were added to the no-host treatment upon harvesting. Two ng of total DNA was used for every sample as a template for the PCR amplifications. DNA quantities were estimated after a natural log transformation of the obtained values. Arbitrary units of *P. infestans* DNA were used.

Cellular responses of *Arabidopsis* to *P. infestans*

To determine the cellular responses of *Arabidopsis* to *P. infestans*, we performed microscope examinations of leaves inoculated at the rosette stage with droplets of zoospores. These analyses revealed the penetration of *Arabidopsis* epidermal cells in multiple independent infection sites. Cyst germination, penetration of epidermal cells and the formation of infection vesicles occurred as early as 46 h after inoculation, and in some cases was followed by the formation of a short secondary hyphae (Fig. 1). Penetrated epidermal cells displayed features typical of the HR, including granulated cell cytoplasm, thickened cell walls, condensed nuclei near the penetration site and autofluorescence under UV light (Fig. 1). These responses were typically limited to the penetrated epidermal cell.

Phytophthora infestans biomass dynamics during infection of *Arabidopsis*

We used kinetic PCR technology to examine changes in *P. infestans* biomass during the interaction with *Arabidopsis* and the host plant tomato (Fig. 2). Primers specific to highly repetitive sequences (> ×10 000) in the *P. infestans* genome were previously used to quantify relative levels of *P. infestans* DNA in infected plant tissue, and were found to reflect an accurate and sensitive estimate of the *P. infestans* biomass (Judelson and Tooley, 2000). We performed kinetic PCR on DNA extracted from discs excised from *Arabidopsis* leaves infected with droplets of *P. infestans* zoospores at successive time points (0, 16, 24, 48 and 72 h after inoculation). Control treatments included inoculated leaves from tomato (host), and inoculum incubated in water in the absence of plant tissue (no-host). In both *Arabidopsis* and tomato, significant increases in the *P. infestans* biomass were observed in the initial 16 h. The *Phytophthora infestans* biomass continued to increase over the 3-day period on tomato, whereas it steadily declined on *Arabidopsis* to reach the lowest level at 72 h after inoculation (Fig. 2). In contrast, no notable changes in biomass were observed over the 3-day period for inoculum

incubated in the absence of plant tissue (Fig. 2). Statistical analyses using ANOVA were performed for each time point and suggested that the changes in biomass observed on *Arabidopsis* are statistically significant ($P = 0.0011$). Based on a protected mean separation, biomass increased significantly during the initial stages of *Arabidopsis* infection followed by a significant decrease in the later stages of the interaction ($P < 0.05$). Independent repetitions of the time course and the kinetic PCR experiments demonstrated that these biomass changes are reproducible (data not shown), however, the extent of the decrease in biomass observed at the later stages varied between experiments.

Local induction of *PR1* and *BGL2* genes during *P. infestans* infection

We used two transgenic *Arabidopsis* lines carrying fusions between the *PR1* and *BGL2* promoters to the β-glucuronidase (GUS) reporter gene (Cao *et al.*, 1997; Manners *et al.*, 1998; Stone *et al.*, 2000) to examine the expression of these defence genes during *P. infestans* infection. The transgenic lines were inoculated at the rosette stage with either *P. infestans* zoospores or water droplets (mock treatment) and the leaves were excised and stained with X-Gluc at successive time points after inoculation. In both lines, elevated levels of *PR*-gene expression was detected 3 days after inoculation, as GUS staining around the inoculation sites (Fig. 3). No GUS expression was detected around mock-inoculated sites. Similar results were obtained from a series of independent experiments. Occasionally, light GUS staining was observed at some sites as early as 2 days after inoculation, but in most cases GUS staining was only observed 3 days or later after inoculation.

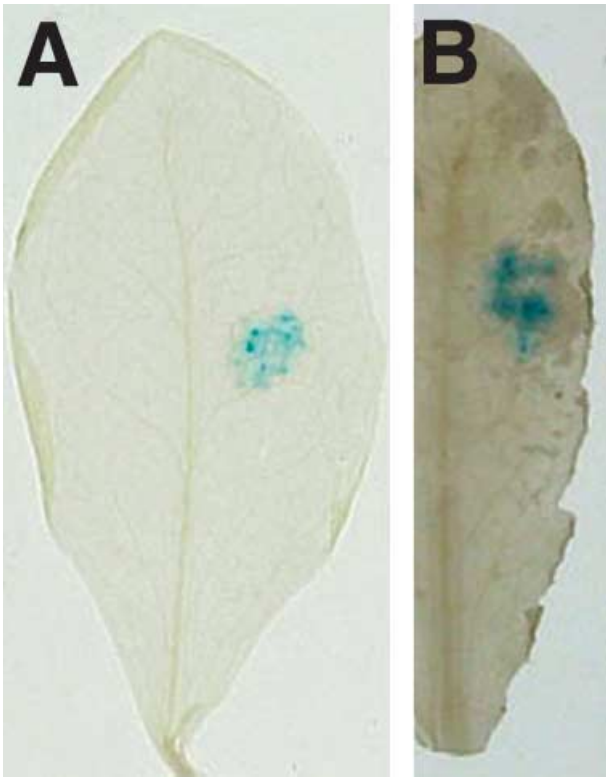

Fig. 3 Induction of *Arabidopsis* (A) *PR1*::GUS and (B) *BGL2*::GUS expression by *Phytophthora infestans*. Transgenic lines were drop-inoculated with *P. infestans* zoospore suspensions. Leaves were harvested and stained with X-Gluc 3 days after inoculation with a droplet of *P. infestans* zoospores. Local expression of GUS was detected in both transgenic lines, 3 days after inoculation. Mock-inoculated sites did not show any detectable staining (left side of leaf in panel A).

Induction of *PDF1.2* during *P. infestans* infection

We assayed the expression of known defence genes during infection of *Arabidopsis* by *P. infestans* using Northern blot time-course analyses. Rosette leaves of *Arabidopsis* plants were sprayed with either water or *P. infestans* zoospore suspensions, and used for RNA extraction at 0, 16, 24, 48 and 72 h after inoculation. Northern blot hybridizations were performed with the defence-response genes *PDF1.2*, *BGL2* and the constitutive gene *UBQ5* (Fig. 4). A significant induction of *PDF1.2* was observed at 48 h after inoculation. No induction of *BGL2* was observed under Northern blot conditions over the 3-day period. No changes in transcript levels were observed in mock-inoculated plants. Independent replications of the time course suggested that the induction of *PDF1.2* is significant and reproducible (data not shown), however, the timing of *PDF1.2* induction varied between 16 and 48 h depending on the experiment.

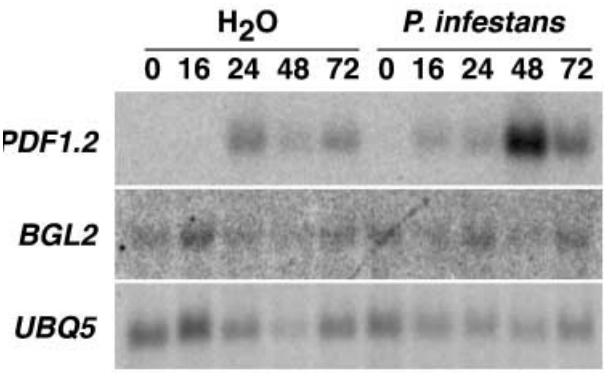

Fig. 4 Time course Northern blot analysis of genes expressed in *Arabidopsis* rosette leaves 0,16, 24, 48 and 72 h after inoculation with *Phytophthora infestans* zoospores or mock inoculation with water. The probes corresponded to *PDF1.2*, a marker gene for the jasmonic acid pathway, and *BGL2*, a marker gene for the salicylic acid-mediated defence pathway. As a loading control, a probe for the constitutive ubiquitin 5 (*UBQ5*) gene was used.

DNA microarray gene-expression profiling of *Arabidopsis* non-host response to *P. infestans*

To further investigate *Arabidopsis* non-host response to *P. infestans*, we used DNA microarray analyses to determine expression profiles for *c.* 11 000 *Arabidopsis* cDNAs using the Arabidopsis Functional Genomics Consortium (AFGC) Microarray Facility (Wisman and Ohlrogge, 2000). In two hybridization experiments, RNA populations derived from mock treated and *P. infestans*-infected leaves were compared against each other using a dye-swap approach. Normalized data were subjected to regression analysis and subsequent outlier detection. Lists of outliers extracted from both hybridization data sets were compared to each other and a list containing an overlapping set of cDNAs was generated. A total of 89 cDNAs were identified that fall outside a 99% confidence interval (CI) in both hybridizations (supplementary data at http://www.oardc.ohio-state.edu/phytophthora/supp.htm). The cDNA identifiers were used to retrieve their respective predicted loci using the TAIR annotation database (http://www.arabidopsis.org) resulting in 54 annotated and non-redundant *Arabidopsis* genes (Table 1). A total of 11 genes were represented by multiple cDNAs (range 2–12) that were identified independently as being differentially expressed in both experiments. This suggests that the experiments and analyses we employed are reliable and robust.

Of the 54 *Arabidopsis* genes identified as differentially expressed during the non-host interaction with *P. infestans*, 52 were up-regulated and 2 were down-regulated. The 54 genes were classified into 7 functional categories that included physiological states related to metabolism, cell wall modification, development, as well as defence and stress responses. Genes that had known functions but that could not be placed in a particular functional category, were compiled in a separate class (Table 1).

Validation of DNA microarray analysis using Northern Blot analysis

We validated the analysis of the microarray data by a regression approach using Northern Blot hybridizations (Fig. 5). A Northern blot containing the RNA samples that were used in the microarray experiment was hybridized with probes from two genes, At1g21400 and At5g25350, that were selected as differentially expressed (Table 1), and two genes, At2g40000 and At3g43740, that were not. In addition, probes for *PDF1.2* and the constitutive gene *UBQ5* were included as controls (Fig. 5). The signals obtained with the various probes were quantified using a phosphor imager, normalized to the *UBQ5* signal, and used to calculate induction ratios. The induction levels obtained by Northern blot and microarray hybridization correlated well (Fig. 5). At1g21400 and At5g25350 were at least twofold induced in the Northern blot experiment vs. 3–5-fold in the microarray

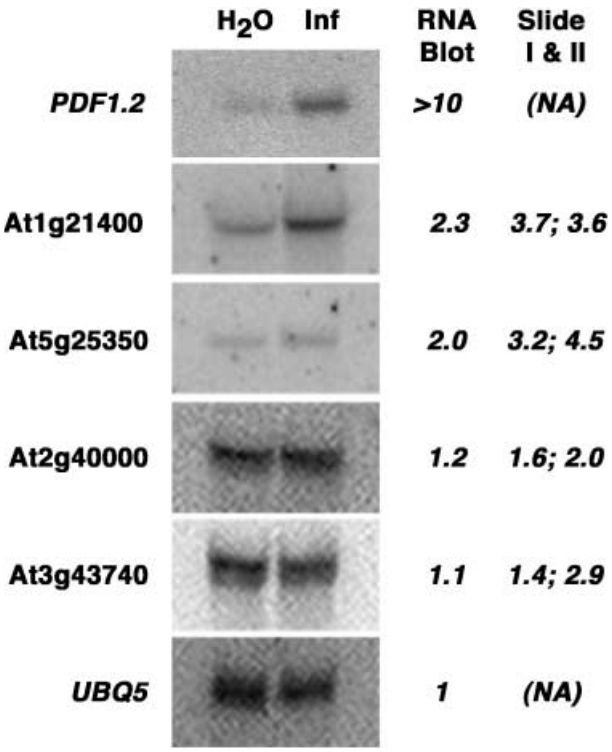

Fig. 5 Validation of microarray analyses using Northern blot hybridization. RNA samples obtained from *Arabidopsis* rosettes 16 h after inoculation with *Phytophthora infestans* (Inf) or mock inoculation with water (H_2O) were blotted and hybridized with probes for At1g21400 and At5g25350, that were selected as differentially expressed based on microarray data analysis, and At2g40000 and At3g43740, that were not selected. *PDF1.2* and *UBQ5* were used as a positive control and a loading control, respectively. The numbers on the right correspond to the induction levels based on the Northern blot (RNA blot), and the two microarray experiments (slides I and II).

hybridizations. In contrast, At2g40000 and At3g43740 showed no significant differential expression by Northern blot hybridization. We also validated the microarray data by Northern Blot analysis using RNA isolated from an independently performed experiment (biological replicate). In this experiment, At1g21400 and At5g25350 were induced 2.8- and 2.4-fold relative to the *UBQ5* gene (data not shown). Overall, these results suggest that the microarray experiment and data processing by regression analyses are reliable.

Comparison of *Arabidopsis* non-host response to *P. infestans* to other defence-related treatments

Transcriptional changes observed during *P. infestans* infection were compared with those reported in two recent microarray analyses of defence-related treatments (Maleck *et al.*, 2000; Schenk *et al.*, 2000). We used tBLASTN searches to compare the data set of differentially expressed genes from our study to those

Table 1 *Arabidopsis* gene loci and number of representative cDNAs found differentially expressed during *Phytophthora* infection by microarray analysis. 11 000 cDNAs were used as targets for labelled cDNA from the two treatments. Genes were selected or considered significantly differentially expressed when the data points fell out of a 99% confidence interval during regression analysis in two separate comparisons (slides I and II). Genes that were found in previous studies (Maleck *et al.*, 2000; Schenk *et al.*, 2000) are indicated.

Putative function	Locus ID	Number of cDNAs	Slide I	Slide II	Identified by	
					Maleck *et al.*	Schenk *et al.*
Stress/defence						
leucine-rich repeats containing protein	At5g25350	1	3.2	4.5		
catalase 3	At1g20620	11	2.2	3.0	yes	yes
peroxidase, putative ATP2a	At2g37130	2	2.8	4.3	yes	yes
glutathione transferase	At2g30860	1	2.4	2.6	yes	yes
glutathione transferase, putative	At4g02520	1	2.2	2.7	yes	yes
family II lipase EXL3	At1g75900	1	2.3	2.6		
lipoxygenase AtLOX2	At3g45140	1	−1.8	−2.3	yes	
thaumatin-like protein	At1g75030	1	−1.3	−1.3	yes	yes
Cell wall modification						
xylosidase (glycosyl hydrolase family 3)	At5g49360	3	2.4	3.1		
xylosidase (glycosyl hydrolase family 3)	At5g64570	1	2.9	4.1		
β-galactosidase (glycosyl hydrolase family 35)	At5g56870	1	2.5	4.7		
galactosidase (glycosyl hydrolase family 35)	At3g13750	2	2.1	2.7		
Development						
ethylene response sensor ERS	At2g40940	1	2.5	3.6		yes
senescence-associated protein SEN1	At4g35770	5	2.2	3.0	yes	yes
dormancy-associated protein, putative	At1g28330	2	2.4	2.7	yes	
auxin-regulated protein	At2g33830	3	2.4	2.8	yes	
cytochrome P450 CYP83B1, indole glucosinolate synthase	At4g31500	2	2.9	4.2	yes	yes
late embryogenesis abundant protein	At4g02380	1	5.0	6.2		
nodulin-like protein	At5g14120	1	2.0	3.1		
Metabolism						
branched-chain amino transferase	At1g10070	1	3.4	3.5		
branched-chain alpha keto-acid dehydrogenase	At1g21400	1	3.7	3.6		
10-formyltetrahydrofolate synthetase	At1g50480	1	2.3	2.8		
acetolactate synthase, putative	At2g31810	1	2.8	2.7		
phytochelatin synthase AtPCS1	At5g44070	1	2.2	3.1		
formate dehydrogenase FDH	At5g14780	1	2.1	3.0		yes
aldehyde dehydrogenase homolog	At1g54100	1	2.2	2.9		yes
putative triosephosphate isomerase	At2g21170	1	2.1	2.2		
molybdopterin synthase sulphurylase	At5g55130	1	2.5	2.1		
glucose transporter	At1g11260	2	2.5	2.2		
Transcription						
AP2 domain protein RAP2.3	At3g16770	3	2.21	3.34	yes	yes
probable transcription regulator protein	At3g48530	1	2.66	3.19		
Other						
glycine-rich RNA binding protein AtGRP7	At2g21660	12	2.39	2.79		yes
putative patatin	At2g26560	1	2.86	5.37		
expressed protein, similar to ubiquitin	At1g26270	1	2.08	2.86		
villin 3 fragment	At3g57410	1	2.7	2.8		
putative myosin heavy chain	At2g32240	1	3.5	4.7		
elongation factor 1-alpha	At1g07940	1	2.5	2.5		
60S ribosomal protein L7A	At3g62870	1	1.9	3.4	yes	
Unknown function						
unknown ORF	At1g31580	1	2.09	2.60		
expressed protein	At5g57655	1	1.92	3.12		yes
expressed protein	At2g36320	1	2.10	3.41	yes	
expressed protein	At3g01290	1	2.66	4.24	yes	yes

Putative function	Locus ID	Number of cDNAs	Slide I	Slide II	Identified by	
					Maleck *et al.*	Schenk *et al.*
expressed protein	At3g15450	1	1.93	2.58	yes	
expressed protein	At1g21680	1	2.05	3.34		
unknown protein	At2g30600	1	2.04	2.44		
unknown protein	At1g73960	1	2.63	3.87		
unknown protein	At1g78110	1	2.08	2.44		
conserved hypothetical protein	At3g24860	1	2.05	3.56		
hypothetical protein	At4g16110	1	1.03	1.38		yes
putative protein; hypothetical protein	At3g49590	1	2.07	2.62		
putative protein	At4g17900	1	2.47	3.42		
putative protein	At4g24690	1	2.05	3.56		
putative protein	At5g53160	1	2.15	4.32		

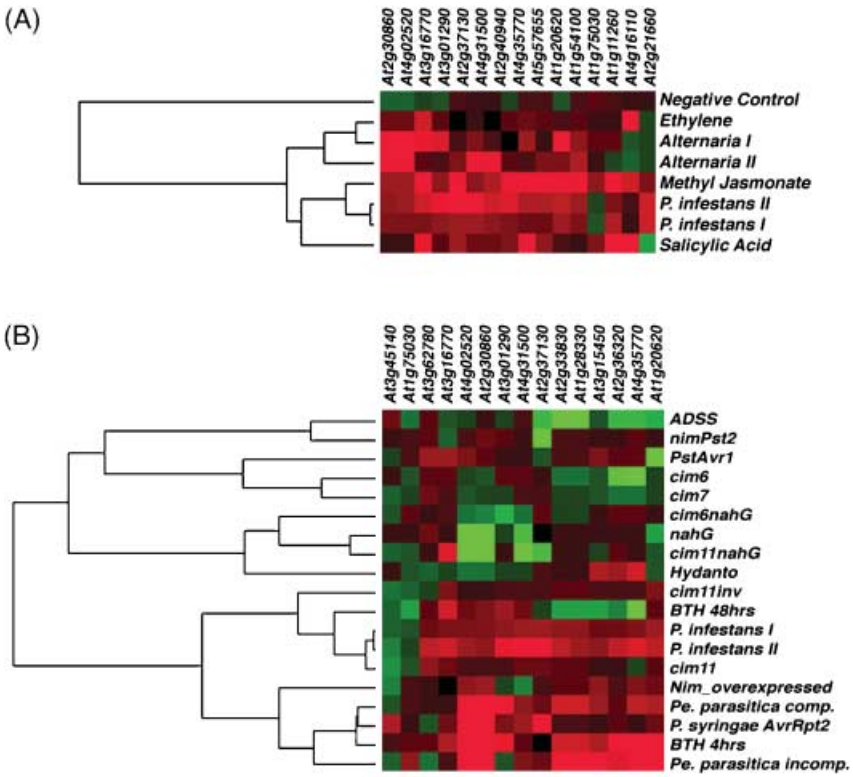

Fig. 6 Cluster analysis illustrating the relatedness of transcriptional changes between *Phytophthora infestans* and other defence-related treatments. The *P. infestans* data sets corresponding to two microarray experiments (*P. infestans* I and II) were combined with overlapping data from the transcriptional profiling experiments reported by (A) Schenk *et al.* (2000) and (B) Maleck *et al.* (2000). The gene numbers are indicated at the top, and the defence treatments were described in Schenk *et al.* (2000) and Maleck *et al.* (2000). The red colour corresponds to up-regulated genes, whereas green represents down-regulated genes.

published previously (see Experimental procedures). Of the 54 *Arabidopsis* genes represented in our data set, 15 matched cDNAs identified in the study performed by Maleck *et al.* (2000) and 15 cDNAs identified by Schenk *et al.* (2000) (Table 1). The expression ratios of the respective genes were extracted from the data sets provided with the two studies and used for building two data matrices. Cluster analysis of the two matrices generated an overview of relatedness between the various treatments and *P. infestans* infection (Fig. 6). Patterns of defence responses induced by MeJA treatment were found to be more similar to responses induced by *P. infestans* infection, whereas ethylene, SA and *Alternaria* inducing conditions resulted in less similar defence-induction profiles (Fig. 6A). Similar comparisons to the SAR-related treatments described by Maleck *et al.* (2000) were made. Cluster analysis revealed a notable resemblance of our expression data to gene expression profiles in *cim11* mutant genotypes, as well as gene expression changes 48 h after treatment with the salicylate analogue benzothiadiazole (BTH). In addition, *P. infestans*

defence responses shared some similarity to those induced by *nim1* over-expression, compatible and incompatible *Peronospora parasitica* interactions, *Pseudomonas syringae* (*AvrRpt2*) infection, and early response after BTH treatment (4 h) (Fig. 6B). Experimental treatments involving plant genotypes containing the *nahG* gene showed least similarity, together with *cim6* and *cim7* and various double-mutant genotypes.

DISCUSSION

We performed the cytological and molecular characterization of a non-host pathosystem involving the model crucifer plant *A. thaliana* and the destructive and economically important oomycete pathogen *P. infestans*. Our cytological observations confirm previous work by Vleeshouwers *et al.* (2000), which showed that the interaction of *P. infestans* with non-host plants, including those that are phylogenetically distant from the solanaceous hosts, is typically associated with the penetration of plant tissue and the HR. However, a more detailed cytological investigation needs to be performed to determine whether the proportion of successful penetration events and the level of HR induction following penetration differ between host and non-host interactions. Nevertheless, our results suggest that recognition of *P. infestans* by *Arabidopsis* takes place and may form one important barrier in non-host resistance. Therefore, a model that evokes an arsenal of *Arabidopsis R* genes that recognize multiple or essential *P. infestans Avr* genes is sufficient to explain non-host resistance in this pathosystem, but it cannot be ruled out that additional layers of nonspecific defence responses occur. With extensive genetic and genomic resources available, the described *Arabidopsis–P. infestans* pathosystem offers excellent prospects for dissecting the complex layers that may form non-host resistance.

In addition to cytological analyses, we used kinetic PCR to monitor relative levels of *P. infestans* DNA and consequent biomass during infection (Fig. 2). Previously, DNA and RNA blot hybridizations have been used to estimate the biomass of pathogenic oomycetes in plant tissue (Kamoun *et al.*, 1998; Rairdan *et al.*, 2001). However, these techniques are not sensitive enough to monitor the small changes in *P. infestans* biomass that are expected to occur on non-host plants. In contrast, kinetic PCR is highly sensitive, quantitative, objective and should prove ideal for non-host pathosystems. To enhance the sensitivity of the kinetic PCR quantification, we used primers corresponding to highly repetitive ($> \times 10\,000$) sequences from the *P. infestans* genome that allow amplification of as little as 10 fg of *P. infestans* DNA (Judelson and Tooley, 2000). The sensitivity of these primers is obvious since we routinely obtained quantifiable signals from inoculation sites bearing as little as 1000 zoospores.

Phytophthora infestans exhibited dynamic changes in biomass over a 3-day infection of *Arabidopsis* (Fig. 2). Over the first 16 h, a significant increase in biomass was observed that was similar to the increase observed on the host tomato. This early increase may correspond to the germination of cysts, penetration of plant epidermis, and formation of infection vesicles and short secondary hyphae, as determined by cytology on both *Arabidopsis* and host plants. This suggests that some level of growth and nuclear division, perhaps in the infection vesicles or secondary hyphae, occurs in *P. infestans* during early infection of *Arabidopsis*. Subsequently, a gradual but significant decrease in *P. infestans* biomass was observed from 24 to 72 h after inoculation of *Arabidopsis*, and contrasted sharply with the steady increase observed on tomato. This decrease may reflect death and degradation of *P. infestans* hyphae caused by the HR and correlates with the termination of pathogen ingress determined by cytology. Interestingly, the dynamic changes in *P. infestans* biomass observed on *Arabidopsis* contrasted with the constant level of biomass observed for *P. infestans* cysts germinating in water in the absence of plant tissue. These results support the interpretation that a successful penetration of the plant epidermis rather than surface growth is required for the biomass increase we observed in the early stages of the interaction. Taken together, these results indicate that *P. infestans* is able to successfully initiate an infection on *Arabidopsis* and complement the cytological analyses.

To gain a first insight into the molecular aspects of *Arabidopsis* non-host response to *P. infestans*, we examined changes in the expression of defence genes using Northern blot hybridizations with probes for *PDF1.2* and *BGL2* (Fig. 4), and the transgenic lines *PR1*::*GUS* and *BGL2*::*GUS* (Fig. 3). Although no induction of *PR1* or *BGL2* was detected by Northern blot analyses, a localized expression of both genes was detected around inoculation sites in the transgenic reporter lines beginning 3 days after inoculation. Since we found *PR1* and *BGL2* to be locally induced at very late stages of the interaction, the discrepancy between the two methods may point to a difference in sensitivity. Considering that most of the cells in the inoculated leaves are not infected, a dilution effect may have reduced the sensitivity of the Northern analysis.

PDF1.2 is a marker for the jasmonate (JA)/ethylene (ET)-mediated defence-response pathways, and its up-regulation has been associated with numerous pathogen or defence-related treatments (Glazebrook, 2001). On the other hand, *PR1* and *BGL2* are marker genes for the salicylate (SA)-mediated defence pathway that is typically induced following infection by necrotizing pathogens or the HR, and during systemic acquired resistance (SAR) (Glazebrook, 2001; Ryals *et al.*, 1996). Taken together, our data suggest the sequential induction of the JA/ET pathway followed by the SA pathway during non-host response of *Arabidopsis* to *P. infestans*. Studies in other *Arabidopsis* pathosystems suggest significant cross-talk and the co-regulation of both SA and JA/ET mediated defence pathways (Clarke *et al.*, 2000; Ellis *et al.*, 2002; Glazebrook, 2001; Schenk *et al.*, 2000). In addition, these pathways have been shown to work antagonistically as well as in concert, to confer enhanced resistance to fungal, bacterial and

oomycete pathogens (Cohn *et al*., 2001; Ellis *et al*., 2002; van Wees *et al*., 2000). The direct role of JA, ET and SA signalling in non-host resistance to *P. infestans* remains to be determined. Quantitative assays using *Arabidopsis* mutant genotypes covering the various branches of known defence pathways are currently underway to address this question.

To gain a better understanding of *Arabidopsis* non-host responses to *P. infestans*, we performed DNA microarray experiments comparing the responses of *Arabidopsis* plants inoculated with *P. infestans* to their mock inoculated counterparts. Despite the harsh selection conditions imposed on the data set, a remarkably high level of redundancy was found amongst the positive cDNAs (see supplementary data at http://www.oardc.ohio-state.edu/phytophthora/supp.htm). Subsequent Northern blot analysis validated our regression analysis strategy. There was a clear correlation in induction levels between the microarray experiment and Northern blot hybridizations using two selected genes and two non-selected genes (Fig. 5). In addition, about one-third of the cDNAs that were identified in our experiments overlapped with cDNAs identified in the defence response gene expression profiling studies of Maleck *et al*. (2000) and Schenk *et al*. (2000) (Table 1).

We classified plant responses using cluster analyses of gene expression profiles across the *P. infestans* treatments and other defence related treatments (Fig. 6). *P. infestans*-induced defence responses were most similar to gene-expression changes after MeJA treatment. From the 15 genes that were used for these comparisons, 14 genes were also induced by MeJA, according to Schenk *et al*. (2000), suggesting that non-host defence responses to *P. infestans* are associated with activation of the JA response pathway (Fig. 6A). In contrast, the expression profiles of all other treatments, such as ethylene, *Alternaria* and SA, had less similarity to our data set. Similarly, patterns of overlap were found in the comparison of SAR-related treatments (Maleck *et al*., 2000) to our data set (Fig. 6B). Cluster analysis of expression profiles indicated a similarity between treatments and the occurrence of two general groups of *Arabidopsis* genes within the set of *P. infestans* up-regulated genes. Two genes were up-regulated 48 h after BTH treatment and had higher expression levels in the *cim11* (constitutive immunity) mutant background. A second and larger group of seven genes was commonly up-regulated in our treatment and during *Peronospora parasitica* compatible and incompatible interactions, BTH treatment (4 h), plants over-expressing *NIM1*, and *Pseudomonas syringae* infection. The transcript levels of the genes used in this comparison were either unchanged or lowered in all NahG plants and *NahG*-containing mutants (*cim11*NahG and *cim6*NahG), *cim6* and *cim7* mutant genotypes and other related treatments.

Some notable genes that are induced in the defence-related treatments examined by Schenk *et al*. (2000) and Maleck *et al*. (2000) were identified in this study (Table 1). Among these, *Rap2.3* (At3g16770), which encodes an AP2 domain transcription factor, was up-regulated during non-host resistance and many other defence responses, suggesting that it may mediate common regulatory steps in defence pathway activation or modulation. Another gene, *Cyp83B1* (At4g31500), is a member of a large family of cytochrome P450 genes, and is involved in the production of indole-glucosinolates as well as the plant hormone IAA (auxin) (Bak and Feyereisen, 2001). The expression of *Cyp83B1* and related members of this gene family was found to be elevated upon SA and MeJA treatments and were associated with an increase in indole-glucosinolates (Mikkelsen *et al*., 2003). Smolen and Bender (2002) identified a non-functional mutant of *Cyp83B1* that showed a lesion-mimic phenotype. Taken together, these data indicate a possible involvement of *Cyp83B1*, and perhaps indole-glucosinolates, in defence responses and possibly regulation of the HR.

Many of the *Arabidopsis* genes identified as up-regulated during non-host response to *P. infestans* could be related to cellular aspects of signalling and defence. For instance, four glycosyl hydrolase genes (At3g13750, At5g49360, At5g56870 and At5g64570), that are possibly involved in modifications of cell wall components were up-regulated two- to fivefold (Table 1). During cell stress and pathogen attack, cell wall modifications are commonly observed (Heath, 1998; Nicholson and Hammerschmidt, 1992; Vleeshouwers *et al*., 2000) which is also illustrated by the cell wall depositions described in Fig. 1. Therefore, cell wall alterations are likely to form a major barrier in non-host resistance. Other notable genes that were up-regulated include genes related to oxidative stress such as catalase (At1g20620), glutathione transferases (At2g30860 and At4g02520), and peroxidase (At2g37130). The occurrence of these genes during non-host HR is not surprising since the role of oxidative stress and production of active oxygen species (AOS) during the HR is well documented (Delledonne *et al*., 2001; Levine *et al*., 1994; Sasabe *et al*., 2000). Since the HR is observed in the *Arabidopsis–P. infestans* interaction, induction of these genes in concert with early defence is plausible. Overall, these data support the concept that defence responses induced by *P. infestans* involve the HR as well as JA-mediated signalling and defence.

In addition to genes in common between non-host and various host defence treatments, some genes uniquely up-regulated during the *P. infestans* interaction were identified (Table 1). The function of these genes in non-host resistance remains unclear, but they represent attractive candidates for functioning in processes unique to non-host resistance to *P. infestans*, and perhaps, other non-host pathogens.

In this study we characterized the interaction between *P. infestans* and the non-host plant *A. thaliana*. An integrated multifaceted approach has enhanced our understanding of this interaction and is helping us to devise future research strategies. Based on the diversity of molecular genetic tools and genomic

resources available for *Arabidopsis* and *Phytophthora*, we expect this non-host pathosystem to become of key importance in studies on molecular plant–microbe interactions. Further research on this pathosystem will provide significant insight into key molecular processes regulating non-host resistance to an economically important pathogen. The knowledge gained will result in immediate biotechnological applications and will offer novel perspectives for engineering durable resistance in crop plants.

EXPERIMENTAL PROCEDURES

Plant growth conditions

Arabidopsis (Col-3) seeds were routinely surface sterilized in 70% EtOH for 30 s, followed by incubation in 50% bleach solution for 10 min. Seeds were then washed multiple times in dH_2O before plating on to MS-Phytagar sucrose plates (1 × MS salts, 2% w/v sucrose, 0.8% w/v Phytagar). Plated seeds were incubated at 4 °C for 3–4 days prior to germination. Seven-to-10 day old seedlings were transferred to potting media and grown under controlled conditions (22 °C, 8 h photoperiod). Mature non-bolting plants at the rosette stage (4–5 weeks) were used for infection experiments.

Phytophthora infestans culturing and infection assays

Cultures of *P. infestans* isolate 90128 (A2 mating type, race 1.3.4.7.8.9.10.11, isolated from potato in the Netherlands in 1990), were routinely grown on rye agar medium supplemented with 2% sucrose (Caten and Jinks, 1968). Zoospores were produced by flooding 11–14-day-old cultures with dH_2O, followed by incubation at 4 °C for 1–3 h. Rosette leaves of 4–5 week-old *Arabidopsis* (Col-3) plants were inoculated with 10 μL droplets of zoospore suspensions for microscopy. For the DNA microarray and Northern time course experiments, complete rosette stage plants were sprayed with zoospore suspensions. Concentrations ranging from 200 000 to 500 000 zoospores/mL were used for all experiments. Deionized water was used as a negative control in all relevant experiments.

Microscope observations

Leaf discs containing the inoculum were excised at various times after inoculation and examined by microscopy for plant response and growth of *P. infestans*. Lactophenol-trypan blue staining and destaining with chloral hydrate were performed as previously described (Colon *et al.*, 1992; Wilson and Coffey, 1980). The discs were examined using a Zeiss Axiophot microscope equipped with a high-pressure mercury vapour lamp. Autofluorescence was observed with a G365 excitation filter, FT395 interference beam splitter and LP420 barrier filters.

GUS staining procedure

Complete *Arabidopsis* leaves were immersed in a GUS staining solution (2 mM 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-Gluc) (Rose Scientific, Edmonton Alberta, Canada) in 0.2% Triton X-100, 50 mM NaHPO4 Buffer (pH 7.2), 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide). A vacuum was applied for 10 min and was then gently released over several minutes. Leaves were incubated at 37 °C for 24 h and examined macroscopically for staining patterns.

Kinetic PCR quantification of *P. infestans*

For kinetic (real-time) PCR quantification of *P. infestans* biomass in plant tissue, four samples were taken for every experimental treatment or time point, with each sample consisting of a pool of four leaf discs corresponding to four independent inoculation sites. Total DNA was extracted from each pool of four leaf discs using a Qiagen Plant DNA extraction kit following the manufacturer's instructions. DNA was quantified using the picogreen ds DNA quantification kit (Molecular Probes, Eugene, OR) and checked by electrophoresis. Dilutions were made using volumes of 10 μL or larger to minimize pipeting errors. Kinetic PCR was performed on a Roche Lightcycler (Roche, Indianapolis, IN) using Lightcycler-FastStart DNA Master SYBR Green I reagents, primers J-08-3 and J-08-4 previously described by Judelson and Tooley (2000), and 2–10 ng of total DNA as a template. PCR conditions were 45 cycles of 1 s at 95 °C, 5 s at 50 °C, and 10 s at 72 °C, with a temperature transition rate of 20 °C per s. *Phytophthora infestans* relative DNA amounts were estimated using a standard curve generated using 100, 10, 1 and 0.1 pg of purified *P. infestans* total DNA. Natural log (ln) transformation was applied on the data to obtain distributions that approached normality. Subsequently, ANOVA was performed on the normalized data using both treatment and replication in the model as fixed effects using the SAS statistical software package version 8 (SAS Institute, Cary, NC). Least significant difference mean separation was used to detect significant differences between the time points assayed.

RNA manipulations and Northern blot analysis

RNA was extracted using the Trizol reagent (Gibco-BRL, Bethesda, MD) using the manufacturer's directions. RNA samples were checked for purity and integrity using spectrophotometry and electrophoresis. When necessary, samples were cleaned further by adding 1 volume of phenol:chloroform:isoamyl-alcohol (125 : 24 : 1, pH 6.7) to the sample, high-speed centrifugation (15800 ***g***, 15 min) and subsequent collection of the aqueous phase. This step was then followed by another extraction using equal volumes of chloroform. RNA was ethanol precipitated from the aqueous phase and dissolved in dH_2O. Membranes for Northern blot

analysis were prepared using a modified method from McMaster and Carmichael (1977) as described in Sambrook *et al.* (1989). 15–20 μg of RNA per sample was used and RNA was blotted on Hybond N^+ membranes (Amersham, Piscataway, NJ) following standard procedures and instructions from the manufacturer.

Hybridization probes were first prepared using Polymerase Chain Reaction (PCR) amplifications. Primers for the amplification of *PDF1.2*, *BGL2* and *UBQ5* were as described elsewhere (Glazebrook *et al.*, 1996; Penninckx *et al.*, 1996; Xiao *et al.*, 2000). Primers for amplification of the selected genes for microarray validation were: At1g21400-F (5′-GAGAAGTCGATATGGACATGATAC-3′) and At1g21400-R (5′-AACGGATGGTGGAGTGAGGAAG-3′); At5g25350-F (5′-CTTCACTCCTACTGATACTACTCA-3′) and At5G25350-R (5′-CTTCGAATTATGTCTGGAATCTTCA-3′); At2g40000-F (5′-CGAAGTTCTCAATTGAGACCAG-3′) and At2g40000-R (5′-GGAACAATCCCAACAAACGGA-3′); At3g43740-F (GAGGAAGATGGTATCATCAG-3′) and At3g43740-R (5′-TCCATTCACGGTGGTTGATG-3′). Amplified fragments were purified from TAE-agarose gels and sequenced using an ABI Prism 377 automated sequencer (PE Applied Biosystems). Similarity searches were used to confirm amplification of the correct fragments.

All probes were labelled with α-^{32}P-dCTP using a random primer labelling kit (Gibco-BRL, Bethesda, MD). All Northern blot hybridizations were carried out at 65 °C in Modified Church Buffer (0.36 M Na_2HPO_4, 0.14 M NaH_2PO_4,1 mM EDTA and 7% SDS). Membranes were washed for 15 min in 1× SSC/0.5%SDS and 0.5× SSC/0.5%SDS at 65 °C, followed by 1 wash in 0.5× SSC/0.5% SDS for 1 min at room temperature. Membranes were exposed to a phosphor imager screen for 24−48 h and scanned using a Molecular Dynamics Storm 840 Phosphor Imager.

DNA microarray hybridizations

Total RNA samples were subjected to standard probe preparations and microarray hybridization procedures as described in the protocol section on the Arabidopsis Functional Genomics Consortium (AFGC) website (http://afgc.stanford.edu/afgc.html/site2.htm). Briefly, two purified mRNA samples were used for synthesis of probes labelled with either CY3 or CY5 fluorescent dye. Four separately labelled cDNA samples were generated using a dye swap to distinguish replicates of the same treatment. These were hybridized to two slides in a dye-swap experimental setup (technical replication). Hybridized slides were scanned using a Scan Array 3000 (GSI Lumonics, Billerica, MA). Two data points were obtained for each spot on one slide.

Microarray data analysis

The generated data were normalized by the default method specified by the AFGC (http://afgc.stanford.edu/afgc_html/site2.htm). All data analysis and processing steps were done using the SAS statistical software package version 8 (SAS Institute, Cary, NC, USA). For analysis purposes, normalized but 'raw' expression values were downloaded for both hybridized slides (technical replicates) from the AFGC web site. The natural log of every expression value was calculated to obtain a data set with distributions that approached normality. Transformed data points were then used for comparisons, applying a regression analysis/outlyer detection technique. In short, for every slide, normalized and transformed expression values of both treatments were plotted against each other (*x*-axis, expression values of mock treatment, *y*-axis, expression values of *P. infestans* treatment). A 99% confidence interval (CI) was generated and used to select cDNAs that correspond to data points falling outside of the 99% CI, representing significant differential expression. Lists of identifiers representing the cDNA spots that were detected were compiled. Only cDNAs identified as outliers at both slides were considered significantly differentially expressed and were used for further analysis. The cDNA identifiers were used to retrieve annotated locus names from the *Arabidopsis* Information Resource (TAIR) database (http://www.Arabidopsis.org). A non-redundant data set was generated and was used to construct cluster data files.

Data compilation and cluster analysis

To identify overlap between the genes identified in this study and published data sets, we first compiled files containing nucleotide sequences for the differentially regulated genes reported in the DNA microarray studies of Schenk *et al.* (2000) and Maleck *et al.* (2000). We then used amino acid sequences corresponding to the 54 differentially expressed genes (Table 1), to perform a TBLASTN search against the generated sequence files. *E*-value scores below 10^{-12} were considered significant. Data matrices were constructed by retrieving and combining expression data of the genes common in either relevant data set. Expression ratios for each gene and treatment were calculated using the inherent control of each treatment as the denominator. Two data matrices were used for cluster analysis using the CLUSTER analysis software package (Eisen *et al.*, 1998). Self-Organizing Map (SOM) analysis was first used to generate classes of genes based on expression ratios across all treatments. Output files generated in this procedure were then used to order the input file for cluster analysis. Complete average linkage hierarchy clustering was used for both comparisons presented. Generated results were visualized and evaluated using TREEVIEW (Eisen *et al.*, 1998).

Microarray data availability

The entire data set can be freely obtained and searched at the AFGC website (http://afgc.stanford.edu/afgc_html/site2.htm). Data for the differentially regulated genes is also provided as a supplementary file (http://www.oardc.ohio-state.edu/phytophthora/supp.htm).

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SUPPLEMENTARY MATERIAL

A Table has been provided, which is available at <http://www.blackwellpublishing.com/products/journals/suppmat/MPP/MPP195/MPP195sm.htm>, and which lists identified cDNAs with their respective GenBank accession and Locus ID numbers. Redundancy represents the number of cDNAs identified from each locus. Ratios S1 and S2 were calculated using the normalized expression values from each hybridized slide.

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