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Molecular and cytological responses of *Medicago truncatula* to *Erysiphe pisi*

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SUMMARY

Powdery mildew is an economically important disease in a number of crop legumes; however, little is known about resistance to the disease in these species. To gain a better understanding of the genetics of resistance and plant responses to powdery mildew in legumes, we developed a pathosystem with *Medicago truncatula* and *Erysiphe pisi*. Screening accessions of *M. truncatula* identified genotypes that are highly susceptible, moderately resistant and highly resistant to the fungus. In the highly resistant genotype, fungal growth was arrested after appressorium development with no colony formation, while in the moderately resistant genotype a small number of colonies formed. Both resistant and moderately resistant genotypes produced hydrogen peroxide and fluorescent compounds at pathogen penetration sites, consistent with a hypersensitive response (HR), although the response was delayed in the moderately resistant genotype. Very little hydrogen peroxide or fluorescence was detected in the susceptible accession. Microarray analysis of *E. pisi*-induced early transcriptional changes detected 55 genes associated with the basal defence response that were similarly regulated in all three genotypes. These included pathogenesis-related genes and other genes involved in defence, signal transduction, senescence, cell wall metabolism and abiotic stress. Genes associated with the HR response included flavonoid pathway genes, and others involved in transport, transcription regulation and signal transduction. A total of 34 potentially novel unknown genes, including two legume-specific genes, were identified in both the basal response and the HR categories. Potential binding sites for two defence-related transcription regulators, Myb and Whirly, were identified in promoter regions of induced genes, and four novel motifs were found in promoter regions of genes repressed in the resistant interaction.

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INTRODUCTION

Plants defend themselves against potential pathogens with an arsenal of preformed barriers and inducible biochemical responses. In gene-for-gene incompatible interactions, specific pathogen avirulence gene products are recognized by plants carrying a corresponding resistance (*R*) gene, leading to localized cell death known as the hypersensitive response (HR) that limits pathogen growth (Nimchuk *et al.*, 2003). An initial oxidative burst provides reactive oxygen species that may be toxic to invaders, act to catalyse structural changes in the plant cell wall, and serve as second messengers in defence signalling. Fluxes in calcium ion levels are critical to activation of the oxidative burst. Other early changes include activation of mitogen-activated protein (MAP) kinases, salicylic acid (SA) signalling, nitric oxide production, and induction of transcription factors (Eulgem, 2005; Klessig *et al.*, 2000). Activation of the SA pathway often leads to long-term disease protection through systemic acquired resistance (SAR) (Durrant and Dong, 2004). The absence of an avirulence gene or appropriate *R* gene leads to a compatible reaction. In this case, plants mount a basal physiological defence response that fails to limit pathogen growth completely.

Large-scale transcript profiling experiments of Arabidopsis have contributed a wealth of data on defence signalling pathways. This research supports two emerging models to explain global gene regulation (Eulgem, 2005; Nimchuk *et al.*, 2003). In the first, pathways engaged by various *R* genes converge to regulate a common repertoire of target genes. In a second, *R* gene pathways and basal resistance control highly overlapping sets of genes. The difference between basal and *R* gene-mediated resistance is in the amplitude and timing of transcription regulation;

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the perceived signal input is greater in incompatible reactions and therefore the output signal is greater (Tao *et al*., 2003).

Medicago truncatula, the barrel medic, is a useful species for identifying genes involved in plant–microbe interactions in legumes. In contrast to many crop legumes it has a relatively small genome size (~500 Mb) and simple diploid genetics. Collaborative research efforts have generated valuable genetic and genomic tools (VandenBosch and Stacey, 2003), and the *M. truncatula* genome sequencing project is nearing completion (Young *et al*., 2005). Comparative mapping studies have demonstrated a high degree of synteny between *M. truncatula* and agriculturally important legume crops, especially *M. sativa* (alfalfa) and pea (Choi *et al*., 2004a,b).

Although powdery mildew has been studied extensively in barley and Arabidopsis, very little is known about the molecular pathology of powdery mildew in legumes. Powdery mildew epidemics can cause significant yield losses in a number of legume crop species. In pea (*Pisum sativum*), severe infections of *Erysiphe pisi* may result in 25–35% yield reductions (Kumar and Singh, 1981). The disease occurs in all parts of the world in which pea is grown (Falloon and Viljanen-Rollinson, 2001). Resistance to *E. pisi* in pea is conditioned by the recessive gene er_1, with a second gene, er_2, identified in some germplasm (Tiwari *et al*., 1997). Because resistance is a recessive trait, only a limited number of cultivars have been developed with resistance to powdery mildew. The two genes appear to condition different responses with er_1 conferring pre-penetration resistance and er_2 post-infection resistance (Fondevilla *et al*., 2006). The fungus has been shown to cause disease on a wide range of crop legumes including alfalfa, *M. truncatula*, common bean (*Phaseolus vulgaris*), faba bean (*Vicia faba*), lentil (*Lens culinaris*), peanut (*Arachis hypogaea*) and red clover (*Trifolium pratense*) (Braun, 1995; Mohan *et al*., 2001) but no information on resistance in these species is available. Powdery mildew screening of accessions by our laboratory and others indicates that *M. truncatula* is a good source of natural resistance (Yaege and Stuteville, 2002). Genetic mapping data suggest that resistance to *E. pisi* is conferred by one dominant major quantitative trait locus (QTL) (C. Ameline-Torregrosa, personal communication).

In barley, the complex *Mla* locus governs powdery mildew resistance, which follows the classic gene-for-gene pattern of fungal avirulence/barley *Mla* gene interaction (Schulze-Lefert and Vogel, 2000). In Arabidopsis, alleles of the neighbouring *RPW8.1* or *RPW8.2* genes control resistance (Xiao *et al*., 2001). In contrast to the barley *Mla* locus, *RPW8*-mediated resistance is broad spectrum in nature. In addition, the RPW8.1 and RPW8.2 protein amino acid sequences are not similar to any of the five classes of *R* genes described so far and may be limited to the Brassicaceae (Xiao *et al*., 2004). In both barley and Arabidopsis hydrogen peroxide is produced rapidly in the incompatible interaction preceding collapse of the attacked epidermal cell (Thordal-Christensen *et al*., 1997; Xiao *et al*., 2001). In Arabidopsis, SA production is required for resistance and SA-induced defences are sufficient for resistance (Xiao *et al*., 2001). Camalexin, the phytoalexin produced by Arabidopsis, is not produced in response to the pathogen (Reuber *et al*., 1998).

Large-scale gene profiling is a powerful tool to uncover both pathways and individual genes involved in a particular process. In this study, a new pathosystem was established using *M. truncatula* and the powdery mildew pathogen *E. pisi*. DNA microarray analysis was used to examine the patterns of gene expression in *M. truncatula* accessions displaying resistant, partially resistant and susceptible phenotypes after inoculation with *E. pisi*. Genes identified as differentially regulated to a similar degree in both resistant and susceptible genotypes were tentatively classified as basal response genes, while those responding to a greater degree in resistant compared with susceptible genotypes were inferred to be associated with the HR. Analysis of genomic sequence from up- and down-regulated genes uncovered conserved promoter sequences that may contribute to the observed transcription accumulation.

RESULTS AND DISCUSSION

M. truncatula/*E. pisi* pathosystem

Three *M. truncatula* genotypes showed distinct disease responses upon inoculation with *E. pisi* (Fig. 1). Complete resistance is represented by the Jemalong/*E. pisi* interaction: no macroscopic symptoms were observed (Fig. 1A). This was confirmed by scanning electron microscopy (SEM) analysis, which showed fungal development arrested once the appressorium developed (not shown). Figure 1B shows infected epidermal cells accumulating autofluorescent defence compounds, characteristic of an incompatible HR. This reaction was first detected at 24 h post-inoculation (hpi). In contrast, the DZA315.16 genotype was highly susceptible to powdery mildew attack (Fig. 1A); infected trifoliate leaves were completely colonized by 7–10 days post-inoculation (dpi). Vigorous hyphal growth was visible microscopically by 24 hpi (pictured in Fig. 1H at 48 hpi) and almost no epidermal cell autofluorescence was observed. The A20 genotype showed moderate resistance both at the macro- and the microscopic level. Patches of mycelium were visible on infected leaves (Fig. 1A), but hyphal growth was restricted (Fig. 1F). Fluorescence microscopy indicated that fungal infection in A20 elicits an HR similar to that in Jemalong at 24 hpi (not shown). The timing of early fungal development up to penetration of host epidermal cells was comparable on each *M. truncatula* genotype, consistent with observations in barley infected with the powdery mildew fungus *Blumeria graminis* (Boyd *et al*., 1995).

The three *M. truncatula* genotypes also showed differences using 3,3-diaminobenzidine (DAB) staining, which detects hydrogen

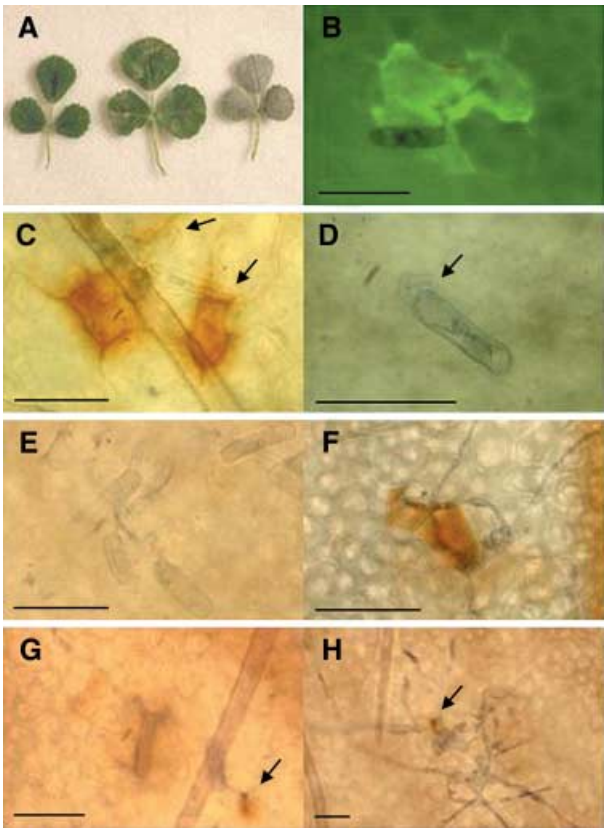

Fig. 1 Reactions of *M. truncatula* genotypes to *E. pisi*. (A) From left to right: phenotypes of Jemalong (resistant), A20 (moderately resistant) and DZA315.16 (susceptible) at 10 days post-inoculation (dpi). (B) Autofluorescent light micrograph of the Jemalong interaction with *E. pisi* at 24 h post-inoculation (hpi). (C) Jemalong light micrograph at 12 hpi showing DAB staining of H_2O_2 accumulation in two epidermal cells. Two infecting *E. pisi* conidia are highlighted by arrows. The tubular structure running diagonal across the image is a leaf hair. (D) Jemalong at 48 hpi. *E. pisi* conidium has developed a multilobed appressorium (arrow) but fails to develop further and in most cases fails to elicit a DAB-positive staining reaction. (E) A20 at 12 hpi. Heavily infected leaf does not show any DAB staining. (F) A20 at 48 hpi. Limited fungal development produces strong whole-cell DAB staining in the epidermis. Amber colour on the right side of the photograph reflects vascular staining. (G) DZA315.16 at 12 hpi. The *E. pisi* conidium on the left has developed an appressorium and a hyphal extension. On the right, the appressorium elicits partial-cell DAB staining (arrow). (H) DZA315.16 at 48 hpi. Developing fungal colony elicits partial-cell DAB staining (arrow); in most cases, infections did not produce any staining. Scale bars = 40 μm.

peroxide (Thordal-Christensen *et al.*, 1997). Resistant genotype Jemalong produced an early, strong response at 12 hpi (Fig. 1C), in which 40% of conidia elicited dark whole-epidermal cell staining. In contrast, the moderately resistant A20 (Fig. 1E) and susceptible DZA315.16 (Fig. 1G) produced very little staining at 12 hpi, with only 2 and 9% of conidia eliciting a DAB-positive response, respectively. For A20, a strong response was detected at a later time point, 48 hpi (Fig. 1F), when 65% of condia elicited staining. Little DAB staining was observed in DZA315.16 at 12, 24 (not shown) or 48 hpi (Fig. 1H); the proportion of conidia associated with staining never exceeded 9%. These data are consistent with an early, *R* gene-mediated oxidative burst during the Jemalong infection, a delayed, yet vigorous response in A20, and a weak, basal reaction in DZA315.16. *E. pisi* growth was halted at the appressorial stage on Jemalong (Fig. 1D), limited development occurred on A20 (Fig. 1F) and small colonies were present on DZA315.16 (Fig. 1H). At this level, the response of Jemalong resembles the prepenetration resistant response of pea accessions with the resistance gene er_1 and A20 resembles accessions with er_2, demonstrating post-penetration resistance (Fondevilla *et al.*, 2006).

Microarray analysis of gene expression

Transcript profiling of host–pathogen interactions was performed using microarrays containing approximately 6000 cDNA clones from libraries representing expressed genes from roots, stems, leaves, flowers and seeds collected during normal growth and under several biotic and abiotic stress conditions (Lohar *et al.*, 2006). Approximately 10% of the genes on the array were previously shown to be involved in plant–microbe interactions. RNA was extracted from leaves collected at 12 hpi in order to detect genes regulated during the DAB-positive oxidative burst (12 hpi) and also those regulated prior to the onset of visible epidermal cell autofluorescence (24 hpi). cDNA from inoculated and control plants was used to assess the expression ratio for each gene (inoculated/control). Examining each experimental data set individually using a two-fold or greater change in expression, the percentage of up-regulated genes was 13, 15 and 5%, and the percentage of down-regulated genes was 9, 10 and 4%, for Jemalong (resistant), A20 (moderately resistant) and DZA315.16 (susceptible), respectively. These ratios are comparable with previous observations of up to 20% genome-wide transcriptional change during basal and *R* gene-mediated resistance in Arabidopsis (Nimchuk *et al.*, 2003). Expression of 13 genes in the Jemalong interaction was verified by quantitative reverse transcription-PCR and the direction of change was found to be the same for all but one gene (Table 1). Microarray data have been deposited at the Gene Expression Omnibus (GEO) repository (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) of the National Center for Biotechnology Information (NCBI) (accession no. GSE6840).

A total of 556 genes showed statistically significant expression ratios in all three genotypes tested using the significance analysis of microarrays (SAM) method (Tusher *et al.*, 2001) and a false discovery rate of 1.5%. As stated above, the majority of genes were not induced or repressed at least two-fold in any genotype in response to *E. pisi* inoculation. A distribution of the ratio/ratios

Table 1 Comparison of quantitative real-time PCR (qPCR) and microarray results.

MtGI*	Annotation	Fold-change†	
		qPCR	Microarray
TC94416	Similar to early nodulin 12 A precursor	56	15.0
TC94309	Similar to xyloglucan-specific fungal endoglucanase inhibitor protein	22	13.8
TC106868	Similar to 2-hydroxyisoflavone dehydratase	45	13.2
TC100534	Similar to patatin-like protein1	54	7.9
TC95600	Similar to calcium/calmodulin-regulated receptor-like kinase	7.2	5.0
TC107445	Similar to GmMYB29B2 protein	10	3.9
TC101591	Similar to EDS1-like protein	1.5	2.0
TC95708	Weakly similar to hypothetical protein T17F15.40 (putative disease resistance protein EDS1)	1.8	2.0
TC96278	Similar to At2g41350/F13H10.10	1.1	0.8
TC94673	Similar to BZIP transcription factor ATB2	1.4	0.5
TC94630	Similar to phaseolin G-box binding protein PG1	0.8	0.5
TC107982	Similar to ERD7 protein	0.2	0.4
TC100816	Alfin-1	0.2	0.4

* *Medicago truncatula* Gene Index tentative consensus (TC) numbers for cDNAs on the microarray (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=medicago).

†Fold change from inoculated Jemalong/control Jemalong at 12 hpi with *E. pisi*. Numbers represent the mean of three qPCR and three microarray experiments.

[(Jemalong inoculated/control)/(DZA315.16 inoculated/control)] showed that most genes were also similarly regulated in both genotypes using a two-fold cutoff (489/556 genes, 88%). We focused on genes that fell into two categories, the basal or compatible defence response (Tables 2 and 3), and the HR (Table 4). The basal response category contains 55 genes that were up- or down-regulated at least two-fold and were regulated to a similar extent (see below) in all three genotypes. The putative HR category contains 41 genes. These showed an incompatible/compatible ratio/ratios of 2.5 or greater, a more stringent cutoff that represents < 10% of the total number. Using the same criteria, only six genes were more highly induced in DZA315.16 than Jemalong (not shown). These results agree with an emerging consensus that the compatible defence response is an attenuated version of the incompatible response. Data from additional infection time points with DZA315.16 might show either a delayed compatible response due to reduced input into the plant signal recognition system (Tao *et al.*, 2003), or an active suppression of compatible host resistance genes, as suggested by work with barley/powdery mildew (Caldo *et al.*, 2004). The remaining 460 genes fell into two additional categories that are not discussed further. The first contained 305 genes that were not induced or repressed at least two-fold in any genotype. The second contained 155 genes in which at least one genotype was induced or repressed by at least two-fold, but the difference between Jemalong and DZA315.16 was less than 2.5-fold. Each gene in the HR and basal response groups was placed in a gene ontology (GO) biological process category (http://www.geneontology.org) based on sequence homologies with genes of known function (Tables 2–4).

Genes induced in the basal defence response

Genes induced to similar levels in resistant, moderately resistant and susceptible genotypes were considered to be part of the basal response to *E. pisi*. The largest group of genes belongs to the GO process response to pest/pathogen/parasite (Table 2). Nine genes are homologous to the pathogenesis-related (PR) proteins known to constitute an early response to a wide variety of pathogens (van Loon, 1999). Five of these belong to the PR10-like family (TC100422, TC106613, TC94217, TC106352, TC106340), and were among the most strongly up-regulated genes in all three genotypes. PR10 proteins are intracellular, acidic proteins that in some cases may possess ribonuclease activity (Bantignies *et al.*, 2000; Borsics and Lados, 2001). Ten potential basal response genes were placed in the GO categories of glutathione metabolism, carbohydrate metabolism, protein metabolism, response to stress and senescence (Table 2). Many of these genes have been previously implicated in pathogen response. Distinct glutathione S-transferases (GSTs) are induced following attack by various pathogens, including powdery mildew fungi (Caldo *et al.*, 2004; Mauch and Dudler, 1993; Reuber *et al.*, 1998). TC107576 and TC106707 encode proteins with strong homology to the ER protein disulphide isomerase precursor, and the TC100382 gene product is homologous with another ER protein, luminal binding protein BiP. In Arabidopsis, expression of these genes precedes PR gene induction (Jelitto-Van Dooren *et al.*, 1999; Wang *et al.*, 2005). Furthermore, the Arabidopsis *BiP2* gene is required for both PR1 secretion and full disease resistance (Wang *et al.*, 2005).

Two *M. truncatula EDS1* genes (TC101591, TC95708) are homologous to the Arabidopsis *EDS1* gene. The expression

Table 2 Genes coordinately up-regulated in response to inoculation with *Erysiphe pisi*.

MtGI*	Annotation	Jem†	A20‡	DZA§	Jem/DZA¶
	Response to pest/pathogen/parasite				
TC100422	Similar to pathogenesis-related protein (Chickpea)	19.3	14.4	14.5	1.3
TC106613	Class 10 PR protein (Alfalfa)	17.3	18.5	14.4	1.2
TC94217	Similar to disease resistance response protein Pi49 (PR10) (Garden pea)	13.7	9.3	12.6	1.1
TC95083	Similar to chitinase (White clover)	7.2	4.7	5.5	1.3
TC106352	Similar to Pprg2 protein (Alfalfa)	5.8	13.5	5.4	1.1
TC106340	Similar to Pprg2 protein (Alfalfa)	4.9	12.2	6.0	0.8
TC100746	Peroxidase 2 precursor (Alfalfa)	4.6	7.4	3.3	1.4
TC101301	Similar to thaumatin-like protein PR-5a (Chickpea)	3.3	2.1	2.0	1.7
TC93997	Similar to pseudo-hevein (Rubber tree)	3.0	2.1	2.4	1.3
TC100682	Similar to thaumatin-like protein precursor Mdtl1 (Apple)	2.4	3.0	2.6	0.9
TC101688	Homologue to pathogenesis-related protein 4A (Garden pea)	2.2	6.0	2.7	0.8
	Glutathione metabolism				
TC95247	Similar to glutathione S-transferase GST 14 (Soybean)	3.9	2.1	2.2	1.8
	Carbohydrate metabolism				
TC106421	Similar to putative endo-xyloglucan transferase (Arabidopsis)	3.2	3.6	2.6	1.2
TC108038	Similar to glucan endo-1 3-beta-D-glucosidase (Chickpea)	3.2	3.8	2.5	1.3
TC103014	Homologue to reversibly glycosylated polypeptide (Cotton)	3.0	3.8	2.0	1.5
	Protein metabolism				
TC100423	Similar to ribosomal protein L15 (Rice)	16.2	15.4	10.7	1.5
TC107576	Similar to disulphide isomerase precursor homologue (Arabidopsis)	2.8	6.7	2.7	1.0
TC106707	Homologue to protein disulphide isomerase precursor (Alfalfa)	2.7	3.9	2.8	1.0
TC100382	Homologue to luminal binding protein 4 precursor (BiP4) (Tobacco)	2.1	4.8	2.5	0.8
	Response to stress				
TC101114	Similar to RD2 protein (Arabidopsis)	2.2	2.0	3.0	0.7
	Senescence				
TC106762	Similar to SRG1 protein (Arabidopsis)	3.4	2.5	2.6	1.3
	Signal transduction				
TC101591	Similar to EDS1-like protein (*Nicotiana benthamiana*)	2.0	6.7	2.3	0.9
	DNA metabolism (DNA binding)				
TC107138	Similar to DNA binding protein S1FA3 (Arabidopsis)	3.8	7.3	2.3	1.7
TC107137	Similar to DNA binding protein S1FA (Spinach)	2.3	2.9	2.2	1.0
	Transport				
TC94641	Similar to Cytochrome P450 (Garden pea)	7.3	7.6	3.3	2.2
TC93969	Similar to ferredoxin III chloroplast precursor (Fd III) (Maize)	2.5	2.7	2.4	1.0
TC108513	Metal transport protein (*M. truncatula*)	2.2	2.3	2.5	0.9
	Unknown/low overlap/weak similarity				
TC109758	Similar to Hsr203J homolog (Garden pea)	5.0	5.3	2.4	2.1
TC102151	Similar to Unknown AT4G15120 (Arabidopsis)	4.8	4.0	2.2	2.2
TC103074	Similar to hypothetical protein F24I3.250 (Arabidopsis)	3.4	4.6	2.5	1.4
TC94295	Similar to CIA2 (Arabidopsis)	2.6	2.7	2.0	1.3
TC102151	Similar to Unknown (Arabidopsis)	2.4	3.0	2.6	0.9
TC94772	Similar to NTGP5 (Arabidopsis)	2.1	2.1	2.0	1.1
TC95708	Weakly similar to disease resistance protein EDS1 (Arabidopsis)	2.0	6.2	2.9	0.7
TC100572	Weakly similar to glutathione S-transferase GST 15 (Soybean)	9.5	5.8	4.5	2.1
TC96147	Weakly similar to unknown protein (Rice)	4.1	3.9	2.1	2.0
TC101996	Weakly similar to pathogenesis-related protein 1 (Arabidopsis)	3.7	4.7	2.7	1.4

*Url: http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=medicago; on each TC webpage, see 6KUG or MTUS library entry for EST clone ID and GenBank accession number.

†Fold change inoculated/control Jemalong (resistant host) 12 hpi with *E. pisi*.

‡Fold change inoculated/control A20 (moderately resistant host) 12 hpi with *E. pisi*.

§Fold-change inoculated/control DZA315.16 (susceptible host) 12 hpi with *E. pisi*.

¶[(Jemalong inoculated/Jemalong control)/(DZA315.16 inoculated)/(DZA315.16 control)].

Table 3 Genes coordinately down-regulated in response to infection with *E. pisi.*

MtGI*	Annotation	Jem†	A20‡	DZA§	Jem/DZA¶
	Response to stress				
TC100454	Homologue to 18.2 kDa class I heat shock protein (Alfalfa)	0.4	0.5	0.3	1.3
TC100459	Homologue to 18.2 kDa class I heat shock protein (Alfalfa)	0.5	0.5	0.4	1.3
TC101359	Similar to ERD4 protein (Rice)	0.5	0.4	0.5	1.0
	Lipid metabolism				
TC106631	Similar to 3-hydroxy-3-methylglutaryl coenzyme A (Garden pea)	0.4	0.3	0.3	1.3
TC106634	Similar to 3-hydroxy-3-methylglutaryl coenzyme A (Garden pea)	0.4	0.3	0.5	0.8
TC94746	Similar to choline kinase CK2 (Soybean)	0.5	0.4	0.5	1.0
	Transcription				
TC94630	Similar to phaseolin G-box binding protein PG1 (Bean)	0.5	0.5	0.3	1.7
	Calcium-mediated signalling				
TC107728	Homologue to putative calmodulin-related protein (Alfalfa)	0.5	0.5	0.4	1.3
	Cell wall organization and biogenesis				
TC107883	Similar to xyloglucan endotransglycosylase 1 (Beech)	0.4	0.4	0.4	1.0
	Flavonoids, stilbene and lignin biosynthesis				
TC100787	CYP81E8 (*M. truncatula*)	0.5	0.5	0.4	1.3
	Senescence				
TC107982	Similar to probable senescence-related protein (Arabidopsis)	0.4	0.4	0.3	1.3
	Unknown/low overlap/weak similarity				
TC95148	Similar to unknown protein (Rice)	0.3	0.3	0.1	3.0
TC101400	Similar to peripheral-type benzodiazepine receptor (Potato)	0.3	0.3	0.2	1.5
TC108642	Similar to 9-*cis*-epoxycarotenoid dioxygenase 4 (Garden pea)	0.4	0.5	0.5	0.8
TC95149	Similar to unknown protein (Rice)	0.4	0.3	0.3	1.3
TC106415	Similar to *Kluyveromyces lactis* protein Q6CQD7	0.5	0.5	0.2	2.5
TC109073	Similarities with *Saccharomyces cerevisiae* GDS1 protein	0.5	0.5	0.5	1.0
TC104426	Weakly similar to putative receptor-like protein kinase (Rice)	0.5	0.5	0.5	1.0

*Url: http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=medicago; on each TC webpage, see 6KUG or MTUS library entry for EST clone ID and GenBank accession number.

†Fold change inoculated/control Jemalong (resistant host) 12 hpi with *E. pisi.*

‡Fold change inoculated/control A20 (moderately resistant host) 12 hpi with *E. pisi.*

§Fold change inoculated/control DZA315.16 (susceptible host) 12 hpi with *E. pisi.*

¶[(Jemalong inoculated/Jemalong control)/(DZA315.16 inoculated)/(DZA315.16 control)].

profiles for both *M. truncatula* genes are very similar; transcript accumulation increases by a factor of two- to six-fold in response to *E. pisi*. Comparable values were obtained for both genes using qPCR (Table 1). Arabidopsis EDS1 is required for *R* gene-dependent resistance to powdery mildew via the SA-mediated EDS1/PAD4 pathway (Falk *et al.*, 1999; Nimchuk *et al.*, 2003). In addition, EDS1 is involved in both basal and non-host resistance (Lipka *et al.*, 2005; Xiao *et al.*, 2005; Zimmerli *et al.*, 2004). In Arabidopsis, three common markers induced by the SA pathway are *PR1, PR5* (thaumatin) and *BGL2* (glucan endo-1,3-beta-glucosidase) (Reuber *et al.*, 1998). These three genes were also up-regulated in our experiments. Two genes related to Arabidopsis *PR1,* TC88317 and TC79410, were induced (3.7-fold and 2.4-fold, respectively) in the Jemalong resistant interaction with *E. pisi* (not shown). As discussed earlier, two thaumatin-like genes, TC101301 and TC100682, were also induced (Table 2). TC108038 (glucan endo-1 3-beta-D-glucosidase, Table 2), which is homologous to Arabidopsis BGL2, was up-regulated between two- and four-fold in all genotypes in response to *E. pisi*. These results suggest that resistance to powdery mildew in *M. truncatula* may be mediated by SA.

The basal pathogen response also includes ten up-regulated *M. truncatula* tentative consensus sequences (TCs) that are annotated in the TIGR MtGI as having weak similarity to other proteins, low overlap to known proteins (< 30%) or homology to proteins of unknown function. Eight of these were also induced in response to another foliar fungal pathogen, *Colletotrichum trifolii* (not shown), providing additional evidence for defence-related functions.

Five genes (TC106352, TC106340, TC101688, TC101591 and TC95708) were more highly up-regulated in A20 than Jemalong using the 2.5-fold ratio/ratios discussed above. The total number of differentially expressed genes in this group was 34 (compared with 47 for Jemalong/DAZ315.16; not shown). This may have

Table 4 Genes coordinately regulated in hypersensitive resistance responses to *E. pisi.*

MtGI*	Annotation	Jem†	A20‡	DZA§	Jem/DZA¶
	Phenylpropanoid metabolism				
TC94281	Isoflavone reductase (*M. truncatula*)	28.0	19.9	10.3	2.7
TC106559	Chain A chalcone synthase (Alfalfa)	26.0	31.0	6.3	4.1
TC100398	Homologue to chalcone reductase (Alfalfa)	15.7	25.5	6.2	2.5
TC106868	Similar to 2-hydroxyisoflavanone dehydratase (Soybean)	13.2	7.1	2.3	5.7
TC100522	Homologue to chalcone-flavonone isomerase 1 (Alfalfa)	12.6	6.1	3.7	3.4
TC106939	Similar to isoflavone synthase (Garden pea)	12.5	7.3	4.5	2.8
TC106539	Homologue to chalcone synthase 2 (Alfalfa)	10.6	8.2	3.8	2.8
TC95934	Homologue to isoflavone-7-O-methyltransferase 9 (Alfalfa)	7.4	7.2	2.6	2.8
TC100839	Similar to cinnamoyl CoA reductase-like protein (Arabidopsis)	4.7	4.2	1.5	3.1
TC100603	Similar to unnamed protein product, contains similarity to chalcone-flavonone isomerase (Arabidopsis)	4.0	3.3	1.5	2.7
	Response to pest/pathogen/parasite				
TC94309	Similar to xyloglucan-specific fungal endoglucanase inhibitor protein (Tomato)	13.8	4.7	1.7	8.1
TC94759	Similar to disease resistance response protein-like AT5G42510 (Arabidopsis)	8.0	4.7	1.7	4.7
TC100534	Similar to patatin-like protein 1 (Tobacco)	7.9	3.7	1.9	4.2
TC100808	Similar to syringolide-induced protein B15-3-5 (Soybean)	6.9	7.8	1.8	3.8
TC94758	Similar to disease resistance response protein-like AT5G42510 (Arabidopsis)	6.3	4.3	1.6	3.9
TC95150	Similar to syringolide-induced protein 13-1-1 (Soybean)	3.9	3.0	1.1	3.5
	Transcription				
TC107445	Similar to GmMYB29B2 protein (Soybean)	3.9	2.5	1.4	2.8
TC94673	Similar to bZIP transcription factor ATB2 (Soybean)	0.5	0.5	1.4	0.4
TC100816	Alfin-1 (Alfalfa)	0.4	0.4	0.1	4.0
	Transport				
TC107302	Similar to putative ABC transporter protein (Arabidopsis)	18.9	6.2	3.4	5.6
TC109829	Similar to mitochondrial phosphate translocator (Arabidopsis)	11.9	2.9	1.8	6.6
	Glutathione metabolism				
TC107601	Similar to glutathione S-transferase GST 13 (Soybean)	11.2	8.0	2.1	5.3
	Signal transduction				
TC95600	Similar to calcium/calmodulin-regulated receptor-like kinase (Alfalfa)	5.0	2.4	1.4	3.6
	Nodulation				
TC100789	MtN1 (*M. truncatula*)	13.3	9.9	3.8	3.5
	Unknown/weak similarity/low overlap/no hit				
TC94715	Similar to putative membrane protein (Potato)	9.1	6.6	1.5	6.1
TC94419	Similar to early nodulin 12 A precursor (N-12 A) (Garden pea)	7.6	2.8	1.7	4.5
TC95464	Similar to unknown (Tomato)	4.5	5.1	1.6	2.8
TC94783	Similar to unknown protein At2g37750 (Arabidopsis)	6.4	12.1	1.4	4.6
TC96321	Weakly similar to putative betaine/proline transporter (Rice)	8.8	2.5	1.4	6.3
TC107427	Weakly similar to O-methyltransferase (Common iceplant)	23.6	15.6	7.3	3.2
TC102075	Weakly similar to sinapyl alcohol dehydrogenase (Poplar)	12.7	12.0	3.2	4.0
TC108027	NA**	6.8	3.6	2.6	2.6
TC104882	NA	5.6	5.4	1.9	2.9
TC107638	NA (Legume-specific)	4.5	5.9	1.5	3.0
TC109813	Similar to oxysterol-binding protein (Arabidopsis)	6.3	2.6	1.5	4.2
TC104326	Weakly similar to expressed protein At2g31090 (Arabidopsis)	6.2	4.7	1.4	4.4
TC95111	Weakly similar to P-glycoprotein-like protein pgp3 (Arabidopsis)	6.6	3.6	1.9	3.5
TC95383	Weakly similar to pathogenesis-related protein PR10A (Durango root)	12.2	5.8	1.9	6.4
TC94416	Similar to early nodulin 12A precursor (N-12 A) (Garden pea)	15.0	3.3	2.3	6.5
TC94417	Similar to early nodulin 12B precursor (N-12B) (Garden pea)	6.8	2.5	1.4	4.9
TC95801	Similar to ferric reductase (Rice)	0.3	0.5	0.7	0.4

*Url: http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=medicago; on each TC webpage, see 6KUG or MTUS library entry for EST clone ID and GenBank accession number.

†Fold change inoculated/control Jemalong (resistant host) 12 hpi with *E. pisi.*

‡Fold change inoculated/control A20 (moderately resistant host) 12 hpi with *E. pisi.*

§Fold change inoculated/control DZA315.16 (susceptible host) 12 hpi with *E. pisi.*

¶[(Jemalong inoculated/Jemalong control)/(DZA315.16 inoculated)/(DZA315.16 control)].

**NA = no significant data available.

some biological significance as the two genotypes are unrelated and therefore the A20 pathogen response may act more quickly/slowly or with greater/lesser amplitude than Jemalong to some signals.

Genes repressed in the basal and HR defence response

Eighteen genes were repressed by *E. pisi* in all three genotypes (Table 3). When Arabidopsis is inoculated with either the non-host barley powdery mildew pathogen or the virulent powdery mildew pathogen *E. cichoracearum*, repressed genes, most of them involved in photosynthesis and general metabolism, account for two-thirds of the differentially expressed genes (Zimmerli *et al.*, 2004). *M. truncatula* genes involved in lipid metabolism and transcription probably fall into this category. Genes in the categories response to stress, calcium-mediated signalling, cell wall organization and biogenesis, flavonoids, stilbene and lignin biosynthesis, and senescence were also down-regulated (Table 3). Each is homologous to genes regulated by biotic and abiotic stress in other systems. TC107982 shares homology with the senescence-related Arabidopsis gene At2g17840. In barley, powdery mildew infection foci suppress senescence, producing 'green islands' among uninfected necrotic tissue (Schulze-Lefert and Vogel, 2000).

Gene expression associated with the hypersensitive response

Genes associated with the HR (Table 4) showed higher transcript accumulation ratios in the resistant compared with the susceptible genotype [(Jemalong inoculated/control)/(DZA315.16 inoculated/control) of 2.5 or greater]. The majority of these genes also had a ratio of 2.5 or greater when comparing expression in A20 (moderately resistant) to DZA315.16. Notably, there was strong up-regulation of genes involved in phyenylpropanoid metabolism. *Medicago* species produce the isoflavonoid phytoalexin medicarpin, via the phenylpropanoid pathway. Table 4 includes seven enzymes (of approximately 11 total) required for synthesis of medicarpin from L-phenylalanine (Dixon *et al.*, 2002). Many of the enzymes in this pathway are encoded by gene families, so the increased expression observed (Table 4) may reflect the contribution of two or more highly related genes. Nonetheless, strong up-regulation was confirmed by qPCR for 2-hydroxyisoflavanone dehydratase (Table 1) and one chalcone synthase (not shown) using gene-specific primers. Induction of phenylpropanoid pathway genes is consistent with the production of autofluorescent products in resistant and partially resistant epidermal cells infected by *E. pisi* (Fig. 1). Previously, medicarpin was shown to inhibit *Colletotrichum trifolii* spore germination and germ tube development in alfalfa (O'Neill and Saunders, 1994) and reduce symptoms following *Phoma medicaginis* attack (He and Dixon, 2000). In barley, resistance to powdery mildew is associated with rapid production of fluorescent phenolic compounds, and inhibition of phenylpropanoid biosynthesis substantially reduces penetration resistance (Zeyen *et al.*, 1995). In contrast, accumulation of autofluorescent products and cell death occurs more than 2 dpi in resistant Arabidopsis (Adam and Somerville, 1996). Although additional experiments are needed to determine the role of phenylpropanoid compounds in resistance to *E. pisi*, the rapid and strong up-regulation of genes in the phenylpropanoid biosynthetic pathway specifically in resistant plants suggests that products of this pathway are involved in limiting *E. pisi* development.

Three potential transcription regulators are associated with the HR to *E. pisi*: an Myb transcription factor (TC107445), a b-ZIP-type transcription factor (TC94673), and a gene encoding a PHD finger family protein (TC100816) originally identified as Alfin-1 (TC100816, Table 4) (Winicov, 1993). Although more than ten Myb-like ESTs were present on the microarray, only TC107445 was induced at least two-fold (Table 4). Interestingly, a second Myb gene, TC94652, was down-regulated in the resistant host, Jemalong, but not in the susceptible host DZA315.16 (not shown). *M. truncatula Alfin*-1 (TC100816) is down-regulated in response to *E. pisi* in all three genotypes. A related gene in Arabidopsis, annotated as Alfin-1, is repressed in response to virulent *Alternaria brassicicola* infection and, to a lesser degree, SA treatment (Schenk *et al.*, 2000). Down-regulation of *M. truncatula* TC100816 was also confirmed in this study by qPCR (Table 1).

Several genes related to nodulins or associated with nodulation were more highly up-regulated in *E. pisi*-resistant genotypes than in the susceptible genotype (Table 4). MtN1 (TC100789) is a small, cysteine-rich protein with homology to pathogen-inducible proteins in pea (Gamas *et al.*, 1998). MtN1 has recently been found to show close similarity to antimicrobial proteins in the defensin family (Silverstein *et al.*, 2005). Although MtN1 was originally proposed to be nodule-specific, it is also induced specifically by *C. trifolii* in resistant *M. truncatula* (Torregrosa *et al.*, 2004). Three genes included in Table 3 (TC94419, TC94416, TC94417) share a short stretch of homology with the pea early nodulins 12A and 12B that encode putative proline-rich cell wall proteins (Govers *et al.*, 1991). Using stringent criteria, TC94416 and TC107638 were identified previously as legume-specific genes (Graham *et al.*, 2004).

Co-regulated promoters contain conserved motifs

Recent work with Arabidopsis has shown that co-regulated genes often share common defence-related promoter elements (Chakravarthy *et al.*, 2003; Chen *et al.*, 2002; Eulgem *et al.*, 2004; Mahalingam *et al.*, 2003; Maleck *et al.*, 2000; Zimmerli *et al.*, 2004). To investigate whether we could identify potential conserved transcription factor binding sites in *M. truncatula*, we collected

Table 5 Conserved *cis*-elements in *M. truncatula* promoters.

Over-represented motifs in induced promoters								
Motif	Element/factor	Query set*	Motif frequency†	Expected frequency‡	Total query§	Number observed¶	Number expected**	*P*-value
yCAyCnACy††	*Myb*II	I	1.00	0.22	14	9	2.8	3.19E-04
		R	0.47	0.22	19	8	3.8	0.02
		U	0.25	0.22	48	10	9.5	0.14
CACGTG	G-box/GBF	I	0.43	0.09	14	6	2.4	0.02
		R	0.21	0.09	19	4	3.2	0.20
		U	0.42	0.09	48	14	8.1	0.01
GCCGCC	GCC box/ERF	I	0.21	0.03	14	2	0.5	0.07
		R	0.05	0.03	19	1	0.6	0.35
		U	0.04	0.03	48	2	1.6	0.26
GTCAAAAw	PB/	I	0.64	0.25	14	8	3.1	3.69E-03
	AtWhy1	R	0.32	0.25	19	5	4.2	0.18
		U	0.46	0.25	48	17	10.5	0.01

*Promoter query sets: I, induced; R, repressed; U, unchanged.
†Average observed motif frequency/1.5 kb promoter.
‡Frequency that each motif is expected to occur by chance/1.5 kb promoter.
§Total number of promoters in query set.
¶Number of promoters containing at least one copy of the motif.
**Number of promoters expected to contain at least one copy of the motif based on a binomial distribution.
††Alternate nucleotide symbols: y(C/T), w(A/T), m(C/A), r(C/T). See text for motif annotations.

81 genomic sequences corresponding to ESTs present on the microarray that fell into one of three query groups: induced genes (I) with an expression ratio of five or greater (14 total), repressed genes (R) with ratios of 0.5 or less (19 total), and a control group (U) of relatively unchanged genes with ratios of 0.7–1.3 (48 total). In most cases, the ratios for genes in each query group were confirmed by similar expression trends in at least one other *M. truncatula* genotype, e.g. Jemalong and A20 showed similar induction ratios. In the induced query group, two related sequences, CCACCnAC and (C/T)CATCAAC, were found to be over-represented (Table 5). These motifs are similar to the ACII sequence (CCACCTACC) in the bean phenylalanine ammonia lyase 2 (*PAL2*) promoter (Hatton *et al*., 1995) and promoters of additional genes coding for enzymes in the phenylpropanoid pathway (da Costa e Silva *et al*., 1993). These motifs (termed Box P) bind Myb transcription factors (da Costa e Silva *et al*., 1993; Feldbrugge *et al*., 1997) and are termed MREs, fitting the type II Myb binding consensus sequence A(A/C)C(A/T)A(A/C)C (Rushton and Somssich, 1998). The *M. truncatula* putative MRE consensus sequence yCAyCnACy (y = C or T) was found 14 times in 14 promoters and was present in at least one copy in 9/14 promoters (Table 5). Myb factors can act both as transcription activators and as transcription repressors (Martin and Paz-Ares, 1997), which might explain why this sequence was also slightly over-represented in the repressed gene promoter set. MREs may exhibit higher activity in combination with the G-box element (CACGTG), which binds bZIP-type transactivators and participates in regulation of genes in response to various environmental signals (Rushton and Somssich, 1998). The G-box sequence was slightly enriched in the induced gene promoter set, occurring in 6/14 promoters, and was found in combination with the MRE in 4/14 promoters.

Whirly transcription factors are single-stranded DNA binding proteins demonstrated to play a role in both basal and *R* gene-mediated defence (Desveaux *et al*., 2005). The Arabidopsis AtWhy1 site, PB, was present here in 8/14 promoters in the induced gene set (Table 5). A similar degree of enrichment was found in Arabidopsis genes co-regulated during SAR (Desveaux *et al*., 2005) and induced in the resistance response to *Peronospora parasitica* (Eulgem *et al*., 2004). The PB site and G-box were slightly over-represented in the unchanged promoter set ($P = 0.01$ for both, Table 5). This might reflect involvement of the two elements in non-defence processes. Alternatively, some genes in the 'unchanged' set may be only weakly up-regulated by these elements (1.2- to 1.3-fold) or may be induced to higher levels at different time points.

Four unique motifs were identified in the repressed query group. The sequence GG(A/C)(A/T)(G/A)AAG is present the highest number of times (21 copies) and was found in 13/19 promoters (Table 6). The AACT(C/T)AAC motif was found 13 times in ten promoters and TTATGATT was found 11 times in eight promoters. The sequence GTGGGTGT is particularly striking because it is found ten times in the down-regulated promoter set (five promoters total) but is not present at all in the induced or unchanged promoter sets. The low *P*-values for GTGGGTGT ($P = 0.004$) and AACT(C/T)AAC ($P = 1.9 \times 10^{-3}$) in the unchanged group actually

Table 6 Over-represented motifs in repressed promoters.

Motif	Query set*	Motif frequency†	Expected frequency‡	Total query§	Number observed¶	Number expected**	*P*-value
GGmwrAAG††	I	0.21	0.22	14	3	2.7	0.25
	R	1.11	0.22	19	13	3.7	4.38E-06
	U	0.46	0.22	48	13	9.4	0.06
TTATGATT	I	0.14	0.29	14	2	1.1	0.21
	R	0.58	0.29	19	8	1.5	3.74E-05
	U	0.21	0.29	48	8	3.7	0.02
AACTyAAC	I	0.29	0.18	14	4	2.3	0.12
	R	0.68	0.18	19	10	3.1	2.40E-04
	U	0.02	0.18	48	1	7.8	1.90E-03
GTGGGTGT	I	0	0.07	14	0	0.9	0.4
	R	0.53	0.07	19	5	1.2	4.70E-03
	U	0	0.07	48	0	3	0.04

*Promoter query sets: I, induced; R, repressed; U, unchanged.
†Average observed motif frequency/1.5 kb promoter.
‡Frequency that each motif is expected to occur by chance/1.5 kb promoter.
§Total number of promoters in query set.
¶Number of promoters containing at least one copy of the motif.
**Number of promoters expected to contain at least one copy of the motif based on a binomial distribution
††Alternate nucleotide symbols: y(C/T), w(A/T), m(C/A), r(C/T). See text for motif annotations.

represent an under-representation of these motifs. Together, 18/19 promoters from this group contain at least one motif discussed above; the average frequency/promoter was 2.9 motifs. The striking difference in the percentage of down-regulated genes in the interaction leading to an HR (9%) compared with the compatible interaction (4%), suggests that gene down-regulation is a component of the resistant interaction. The functionality of the motifs identified in the repressed query group and the role of the repressed genes, most of unknown function, remains to be tested.

EXPERIMENTAL PROCEDURES

Plant material

Three *M. truncatula* genotypes were used for *E. pisi* inoculations. Jemalong J6 is derived from the cultivar Jemalong, DZA315.16 is derived from a naturally occurring Algerian population (Thoquet *et al.*, 2002) and A20 has been used in mutagenesis and mapping experiments (Choi *et al.*, 2004a; Penmetsa and Cook, 2000). Jemalong J6 and A17 have essentially identical genotypes (Thoquet *et al.*, 2002).

Pathogen inoculation

Surface-sterilized *M. truncatula* seeds were vernalized at 4 °C for 36 h, and then transferred to water-soaked Jiffy-7 peat pellets (Jiffy Product of America, Norwalk, OH) that were placed inside clay pots containing SB-300 soil mix (Sungrow USA, Pine Bluff, AR). A pure culture of *E. pisi* that was established from a naturally infected pea plant was obtained from Dr Tim L. W. Carver, Department of Environmental Biology, Institute of Grassland and Environmental Research, Plas Gogerddan, Aberystwyth, Ceredigion SY23 3EB, Wales, UK. Cultures were maintained on Perfection 8771 pea plants grown in isolation at 22 °C with a 16-h photoperiod (300 μmol/min/m^2) and 8 h of darkness at 16 °C. Four-week-old *M. truncatula* plants with two to three fully expanded compound leaves were inoculated with approximately 300–500 spores/cm^2 leaflet area using a 180-cm settling tower. After 20 min, plants were returned to the growth chamber.

Microscopy of *E. pisi*-treated leaves

Inoculated leaves were excised and immersed in ethanol/acetic acid (3 : 1 v/v) for 24 h to fix and bleach the tissues. Leaf segments were gently washed twice in water and then stored in lactoglycerol (lactic acid/glycerol/water; 1 : 1 : 1) containing 0.1% sodium azide. Prior to observation, leaf segments were stained with a drop of lactoglycerol/0.1% trypan blue. Spore germination and appressorial contacts were observed using transmitted bright field microscopy. Autofluorescent responses in epidermal cells were visualized by switching to incident fluorescence microscopy (blue exciter filter, maximum transmittance 400 nm; dichroic mirror and barrier filter transmittance range > 510 nm) (Zeyen *et al.*, 1995). DAB staining was performed essentially as described by Thordal-Christensen *et al.* (1997) except that leaves were incubated in DAB solution for 10–12 h in the dark. Following DAB treatment, leaves were stained for 1 h in 40–50 °C LPTB

[1 : 1 : 1 : 1; lactic acid, phenol, glycerol, trypan blue solution (10 mg/mL in water)], rinsed and stored in lactoglycerol. Counts of conidiospores associated with whole-epidermal DAB staining were obtained from at least 60 conidia from at least six leaflets from two independent experiments. Images were captured using a Nikon Coolpix 990 camera attached to an Olympus BX50 photomicroscope. In some cases, images from two focal planes were superimposed using Adobe Photoshop 5.0 software.

Microarray analysis of gene expression

Each microarray slide contained 6144 ESTs chosen from a wide variety of cDNA libraries representing roots, stems, leaves, flowers and seeds collected during normal growth and under several biotic and abiotic stress conditions. Details of the slide fabrication and processing have been described (Lohar *et al*., 2006). A total of 4815 EST clones passed a resequencing test designed to verify clone identity; sequence data for each of these have been deposited in GenBank (Lohar *et al*., 2006). From 4815 ESTs, 4600 individual TCs and 117 singletons are represented for a total of 4717 potentially unique genes. The gene expression ratios obtained using microarray slides and the data analysis in this study have been authenticated previously using independent methods (Lohar *et al*., 2006).

Fully expanded leaves from plants inoculated with *E. pisi* and non-inoculated control plants were collected 12 hpi and then subjected immediately to RNA extraction or alternatively, flash-frozen and stored at –80 °C. Leaf samples from three independent biological replicates were collected for the experiment. Approximately 100 mg of frozen trifoliate leaf was processed using Qiagen RNeasy Plant Mini Kits (Qiagen Inc., Valencia, CA) with slight modifications. The volumes of RLT buffer and ethanol used in first and second steps, respectively, were doubled, and subsequent steps were performed as described in the kit handbook. cDNA synthesis and labelling were done as described previously (Lohar *et al*., 2006). Hybridizations were done using a 'dye-swap' design in which each cDNA was labelled separately with either Cy3 or Cy5 and combined with its matching pair. Three separate slides were hybridized with each dye-swap combination making a total of six slides for each experiment. Hybridization, washing, slide scanning, and normalization were done as described previously (Lohar *et al*., 2006). In each experiment, a stringent threshold for acceptance was set so that the false discovery rate ranged from < 1% to 1.4% (Tusher *et al*., 2001).

Quantitative reverse-transcription PCR

RNA was prepared as described above. Each sample was treated with RQ1 RNAse-free DNAase (Promega Corp., Madison, WI), and purified again using the Qiagen RNeasy Plant Mini Kit RNA cleanup protocol. cDNA was synthesized from 1 mg total RNA using the iScript cDNA synthesis kit (BIO-RAD, Hercules, CA). PCR reactions used BIO-RAD iTaq SYBR Green supermix with ROX kit according to the manufacturer's instructions in the ABI Prism 7000 Sequence Detection System from Applied Biosystems (Foster City, CA) using the following regime: 2 min at 95 °C, then 40 cycles at 95 °C for 15 s and 55 °C for 1 min. qPCR reactions were performed in triplicate for each of three biological replications and each number in Table 1 represents the mean value. Primers used to amplify individual genes are listed in supplementary Table S1. Data analysis and calculations for comparing expression data were performed using the $\Delta\Delta$CT (threshold cycle) method. Each gene/18S primer combination was tested for equal amplification efficiency. The amplification specificity for each gene was confirmed by a single dissociation curve peak generated at the end of the PCR cycle.

Promoter analysis

Jemalong ESTs with induction ratios of at least five-fold and repression ratios of at least two-fold in response to *E. pisi* were selected for promoter analysis. As a control group, 48 genes that passed SAM analysis with ratios close to 1 (0.7–1.3) were chosen as relatively unchanged in response to *E. pisi.* The corresponding TC sequence for each EST was used for BLASTN analysis against the non-redundant sequence database (nr) at GenBank. BAC sequences with > 98% identity were collected, corresponding coding regions were predicted using FGENESH software (http://www.softberry.com), and the 1.5-kb sequence upstream of the first ATG in the open reading frame was used for further analysis. Potential over-represented motifs were identified by searching the four groups of promoters using (1) > 20 published motifs associated with plant defence, (2) 803 regulatory motifs present in the Softberry RegSite PlantDB database (http://www.softberry.com) and (3) the MotifSampler program (http://www.esat.kuleuven.ac.be/~thijs/Work/MotifSampler.html), which identifies potentially novel motifs. The expected frequency of each motif was calculated using the average G+C content of 30% observed in these promoters. Statistical analysis was performed assuming a binomial distribution as described previously (Maleck *et al*., 2000).

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SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article:

Table S1 Primers used in real-time PCR.

This material is available as part of the online article from:
http://www.blackwell-synergy.com/doi/full/
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