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Arbuscular mycorrhizal symbiosis is accompanied by local and systemic alterations in gene expression and an increase in disease resistance in the shoots

Jinyuan Liu[1,†], Ignacio Maldonado-Mendoza[1,‡], Melina Lopez-Meyer[1,‡], Foo Cheung[2], Christopher D. Town[2] and Maria J. Harrison[1,*]

[1]*Boyce Thompson Institute for Plant Research, Cornell University, Tower Road, Ithaca, NY14853, USA, and*
[2]*The Institute for Genomic Research, 9712 Medical Center Drive, Rockville, MD 20850, USA*

Received 22 November 2006; revised 11 January 2007; accepted 12 January 2007.
*For correspondence (fax +1 607 254 6779; e-mail mjh78@cornell.edu).
†Present address: Department of Plant Pathology, 334 Plant Science Building, Cornell University, Ithaca, NY14853, USA.
‡Present address: CIIDIR-IPN Unidad Sinaloa, Blvd. Juan de Dios Bátiz Paredes 250, Guasave, Sinaloa, México, CP 081101.

Summary

In natural ecosystems, the roots of many plants exist in association with arbuscular mycorrhizal (AM) fungi, and the resulting symbiosis has profound effects on the plant. The most frequently documented response is an increase in phosphorus nutrition; however, other effects have been noted, including increased resistance to abiotic and biotic stresses. Here we used a 16 000-feature oligonucleotide array and real-time quantitative RT-PCR to explore transcriptional changes triggered in *Medicago truncatula* roots and shoots as a result of AM symbiosis. By controlling the experimental conditions, phosphorus-related effects were minimized, and both local and systemic transcriptional responses to the AM fungus were revealed. The transcriptional response of the roots and shoots differed in both the magnitude of gene induction and the predicted functional categories of the mycorrhiza-regulated genes. In the roots, genes regulated in response to three different AM fungi were identified, and, through split-root experiments, an additional layer of regulation, in the colonized or non-colonized sections of the mycorrhizal root system, was uncovered. Transcript profiles of the shoots of mycorrhizal plants indicated the systemic induction of many genes predicted to be involved in stress or defense responses, and suggested that mycorrhizal plants might display enhanced disease resistance. Experimental evidence supports this prediction, and mycorrhizal *M. truncatula* plants showed increased resistance to a virulent bacterial pathogen, *Xanthomonas campestris*. Thus, the symbiosis is accompanied by a complex pattern of local and systemic changes in gene expression, including the induction of a functional defense response.

Keywords: arbuscular mycorrhizal symbiosis, microarray, *Medicago truncatula*, roots, defense response, induced systemic resistance.

Introduction

Most vascular flowering plants have the ability to form mutualistic associations with soil fungi from the Glomeromycota (Smith and Read, 1997). The resulting symbiosis is called an arbuscular mycorrhiza (AM), and it occurs widely in terrestrial ecosystems throughout the world. The symbiosis develops in the roots where the fungus colonizes the cortex and obtains carbon from the plant while facilitating the transfer of mineral nutrients to the root cells (reviewed by Harrison, 2005). In the AM symbiosis, the fungus invades the cortical cells and forms differentiated hyphae, called arbuscules, within the cells. As each arbuscule develops, the plant cell envelops it in a novel membrane, and the resulting symbiotic interface is equipped with unique transport specificities and functions centrally in nutrient exchange (Bonfante-Fasolo, 1984; Smith and Smith, 1989). Arbuscules are relatively short-lived structures, and development and decay of arbuscules is ongoing within the root at the cellular level (Alexander *et al.*, 1988). However, at the whole-plant level, the AM symbiosis is stable and can be maintained throughout the

life of the plant (Gianinazzi-Pearson, 1996; Harrison, 2005; Smith and Read, 1997).

There is substantial interest in understanding the molecular basis of the AM symbiosis, and, through forward genetic screens, five genes essential for development of the AM symbiosis have been identified (Ane *et al.*, 2004; Endre *et al.*, 2002; Levy *et al.*, 2004; Mitra *et al.*, 2004; Stracke *et al.*, 2002; Imaizumi-Anraku, 2005; Kanamori, 2006). They encode signal transduction components and are also required for the formation of nitrogen-fixing symbiosis with rhizobia, which indicates commonalities in the signalling pathways operating in these two symbioses (Parniske, 2004). A variety of approaches have been used to document the transcriptional responses that occur in the roots during development of an AM symbiosis. In particular, whole-genome transcript profiling in rice (*Oryza sativa*), and partial-genome transcript profiling in *Medicago truncatula*, have provided insights into the mycorrhiza-responsive segments of these transcriptomes (Guimil *et al.*, 2005; Hohnjec *et al.*, 2005; Kuster *et al.*, 2004; Liu *et al.*, 2003). Initial comparative analyses revealed some similarities in the transcriptional responses of monocots and dicots to AM fungi (Guimil *et al.*, 2005). Further studies of individual genes have shown that some mycorrhiza-induced genes are expressed only during the AM symbiosis, while others are constitutively expressed in roots and are induced in the AM symbiosis. A subset of these are regulated as a consequence of alterations in the phosphate status of the plant (Brechenmacher *et al.*, 2004; Guimil *et al.*, 2005; Hohnjec *et al.*, 2005; Journet *et al.*, 2002; Kuster *et al.*, 2004; Liu *et al.*, 2003; Manthey *et al.*, 2004; Weidmann *et al.*, 2004; Wulf *et al.*, 2003). In addition, the mycorrhiza-regulated genes display a variety of spatial expression patterns suggesting the existence of multiple regulatory pathways (Hohnjec *et al.*, 2005; Liu *et al.*, 2003; Manthey *et al.*, 2004; Wulf *et al.*, 2003). In rice, 40% of the AM-regulated genes were differentially expressed in response to an interaction with one of two fungal pathogens, *Magnaporthe grisea* or *Fusarium moliniforme*, indicating an overlap in the responses to beneficial and pathogenic fungi (Guimil *et al.*, 2005; Paszkowski, 2006). In *M. truncatula*, extensive overlap in the transcriptional responses to AM fungi and to *Sinorhizobium meliloti* has been observed (Kuster *et al.*, 2004; Manthey *et al.*, 2004). Furthermore, similarities in the initial responses of *M. truncatula* to an AM fungus, *Glomus mosseae*, and a fluorescent pseudomonad, *P. fluorescens*, have been noted, with 10 genes showing induction in both interactions (Sanchez *et al.*, 2005).

Although the physical interaction between the AM symbionts occurs in the root cortex, the physiology of the whole plant has an impact on development of the symbiosis, and, in turn, the AM symbiosis alters the physiology of the whole plant. Split-root experiments and foliar feeding experiments indicate that colonization of the root system is suppressed in response to high phosphate (P_i) levels, and that this is mediated by a systemic signal that probably originates in the shoots (Braunberger *et al.*, 1991; Graham *et al.*, 1982; Menge *et al.*, 1978; Sanders, 1974; Schwab *et al.*, 1983, 1991; Thomson *et al.*, 1991). There is also evidence for long-distance signals unrelated to the nutritional status of the plant. In conditions under which P_i levels were equalized, colonization of one half of a root system resulted in suppression of colonization of the remaining half of the root system, suggesting the presence of a mobile, auto-inhibitory signal (Vierheilig, 2004; Vierheilig *et al.*, 2000). This is reminiscent of the rhizobium–legume symbiosis where auto-regulation of nodule number occurs in response to a systemic signal originating in the shoots (Schnabel *et al.*, 2005; Searle *et al.*, 2002).

In many plants, challenge with a pathogen induces systemic acquired resistance (SAR) and the plant displays increased resistance to subsequent pathogen challenge (reviewed in Durrant and Dong, 2004). Colonization with non-virulent bacteria, such as fluorescent pseudomonads, also affects the whole plant and induces a state termed 'induced systemic resistance' (ISR), where plants show increased resistance to subsequent challenge by certain pathogens (Pieterse *et al.*, 1996). Likewise, development of the AM symbiosis has been observed to alter subsequent interactions with other organisms. Split-root experiments with tomato (*Solanum lycopersicum*) and *Glomus mosseae* revealed a systemic bioprotective effect in the roots against challenge with *Phytohpthora parasitica* (Cordier *et al.*, 1998; Pozo *et al.*, 2002). In the non-mycorrhizal portion of the mycorrhizal root system, cell-wall thickening was noted adjacent to *P. parasitica* hyphae, and hyphae that invaded root cells were encased in papilla-like structures and development was arrested. These responses were not observed in non-mycorrhizal control plants (Cordier *et al.*, 1998). Bioprotective responses to other pathogens have also been noted, including enhanced resistance to root-knot nematodes (Akkopru and Demir, 2005; Berta *et al.*, 2005; Li *et al.*, 2006). In contrast, mycorrhiza-associated responses that impact pathogens invading the aerial portions of the plant have received little attention, but some effects have been observed. In mycorrhizal tobacco plants (*Nicotiana tabacum*) challenged with *Botrytis cinerea*, the mycorrhizal treatment resulted in a delay in the accumulation of PR proteins and an increase in disease symptom severity (Shaul *et al.*, 1999).

Previously, as part of a program to examine the molecular basis of the AM symbiosis, we generated ESTs from *M. truncatula* roots in association with *Glomus versiforme* and used transcript profiling to identify mycorrhiza-induced genes. Here we use a combination of transcript profiling with a 16 000-feature oligonucleotide-based array and real-time quantitative RT-PCR, to explore the transcriptional changes that are triggered systemically in the roots

and shoots as a result of AM symbiosis. The results indicate a complex pattern of local and systemic changes in gene expression in the roots. Furthermore, we found significant alterations in the transcript profile of the shoots. Coupled with additional experimental data, we suggest that the AM symbiosis triggers systemic changes in gene expression that increase disease resistance of the plant.

Results

Transcript profiles in roots and shoots of M. truncatula *colonized with* Glomus intraradices

The goals of these profiling studies were to expand our understanding of the transcriptional responses of roots to colonization by AM fungi, and to determine whether development of a mycorrhizal symbiosis causes alterations in the transcriptional profile of the shoots other than those associated with alterations in phosphorus nutrition. The experiments were designed to avoid a significant enhancement of phosphorus nutrition so that other, possibly minor, transcriptional changes might be uncovered. *M. truncatula* plants were inoculated with *G. intraradices* and grown under conditions that permit robust development of the AM symbiosis, but under which a mycorrhiza-induced growth response, or significant increase in P_i nutrition, does not occur. Under these conditions, at 30 days post-inoculation (dpi), the shoot P_i content and mass of the *M. truncatula*/*G. intraradices* plants were comparable to those of the mock-inoculated controls (Table 1). Three biological replicate experiments were established, and RNA was prepared from the roots and shoots and used to probe a *M. truncatula* 16 K (16 000-feature) oligonucleotide array. This revealed 545 genes whose transcript levels increased in *M. truncatula*/*G. intraradices* roots, and 102 genes with reduced transcript levels (Figure S1 and Table S1). Of these 647 genes, 161 have previously been reported as differentially regulated in *M. truncatula/G. intraradices* roots (Hohnjec *et al.*, 2005). Significant changes in gene expression were also detected in the shoots of *M. truncatula/ G. intraradices* plants, and 468 genes showed a significant increase in transcript levels and 131 showed a significant decrease (Figure S1 and Table S2). Genes expressed in shoots in response to the AM symbiosis have not been reported previously. Consequently, we validated a subset of the data by quantitative RT-PCR. Fifty-two upregulated genes and 16 downregulated genes, representing a range of biological functions and including genes with high and low expression levels, were selected for evaluation. Using RNA from one of the biological replicate experiments used in the array hybridization, the expression patterns of 62 of the 68 genes were confirmed. Subsequently, the expression of a subset of these genes was assayed in an RNA sample independent of that used for the arrays, and the expression patterns of 14 of 16 genes examined were confirmed (Table 2).

Table 1 Colonization level, fresh weight and phosphorus (P) content of shoot tissues of mycorrhizal and mock-inoculated *M. truncatula* plants

Replicates	RLC (%)	Roots (g)	Shoots (g)	Shoot *P* (nmol g^{-1}FW)
Experiment 1: *M. truncatula*/*G. Intraradices*[a]				
Mt-1	0	19.8	6.5	19.3 ± 2.2
Mt-2	0	18.3	6.1	21.5 ± 2.0
Mt-3	0	23.0	7.6	22.3 ± 3.0
Mt/Gi-1	49	20.8	7.9	23.4 ± 1.1
Mt/Gi-2	50	17.8	7.0	22.1 ± 1.2
Mt/Gi-3	53	21.0	8.7	20.5 ± 1.8
Experiment 2: *M. truncatula*/*Gigaspora gigantea*[b]				
Mt-a	0	2.2	1.4	13.7 ± 1.7
Mt-b	0	2.5	1.4	15.2 ± 2.4
Mt-c	0	1.6	1.1	14.5 ± 1.0
Mt/Gigi-a	39	2.6	1.4	15.2 ± 1.3
Mt/Gigi-b	47	2.4	1.0	14.7 ± 1.9
Mt/Gigi-c	44	2.0	1.5	14.4 ± 1.2

[a]Experiment 1, two-week-old *M. truncatula* plants were transplanted in 11 cm pots (eight plants per pot) and inoculated with *G. intraradices* spores. Three replicate experiments were prepared. Mt-1 to Mt-3 indicates mock-inoculated replicates; Mt/Gi indicates replicates inoculated with *G. intraradices*. The plants were harvested at 30 dpi.
[b]Experiment 2, three-day-old *M. truncatula* seedlings were transplanted in small cones (3 × 15 cm, 10 plants per cone) and inoculated with *Gigaspora gigantea* spores. The plants were harvested at 28 dpi. Three replicate experiments were prepared. Mt-a to Mt-c indicate mock-inoculated replicates; Mt/Gigi indicates replicates inoculated with *Gigaspora gigantea*.

Using BLASTX and the Arabidopsis Information Resources Slim Gene Ontology (GO) protein databases (http://www.tigr.org/tigr-scripts/tgi/GO_browser.pl?species), the genes induced in mycorrhizal root and shoot tissues were grouped into 16 different functional classes (Figure 1). This revealed differences in the transcriptional responses in the two tissues. The percentage of genes in the hydrolytic activity, enzyme activity and structural molecular activity categories is greater among the genes induced in roots relative to those induced in the shoots. Surprisingly, genes involved in transcriptional regulation form a larger proportion of the genes induced in the shoots. In addition, there is a difference in the representation of genes that encode proteins that do not have an Arabidopsis homolog. The proportion is slightly higher in the group of genes differentially expressed in the roots. For each gene in this category (no Arabidopsis homolog), we used BLAST analyses to determine whether there was a possible rice ortholog. For 21 of the 165 root-responsive genes and 14 of the 117 shoot-responsive genes, it was possible to identify a rice ortholog (Table S3). As rice forms AM symbioses, while Arabidopsis does not, it is possible that these genes encode proteins specialized for the AM symbiosis.

Table 2 Sixty-eight genes showing differential expression in *M. truncatula*/*G. intraradices* shoots validated by quantitative RT-PCR

			Mt/Gi shoot versus Mt shoot		
TIGR ID	Oligo ID	Putative annotation	Array ratio	*P*-value	Quantitative RT-PCR ratio[a]
Upregulated genes					
TC94037	MT007045	RING zinc finger protein-like	2.77	1.5E-03	**2.08**
TC94336	MT007207	NADH-plastoquinone oxidoreductase chain1	2.63	9.1E-05	**2.60/2.07**[b]
TC94430	MT015842	Functional candidate resistance protein KR1	2.64	5.6E-05	**1.75**
TC94571	MT000437	Pheromone receptor-like protein	4.32	2.6E-04	**3.18/3.95**
TC94597	MT000368	SRC2	2.79	1.1E-04	**2.43**
TC94630	MT000453	Phaseolin G-box binding protein PG1	2.00	2.9E-06	**3.57**
TC94758	MT015417	Disease resistance response protein	2.09	1.3E-03	**2.40/2.84**
TC94854	MT007574	Syringolide-induced protein 14-1-1	4.09	8.4E-06	**5.00**
TC94874	MT007607	Transcription factor WRKY32	4.62	7.0E-04	**3.62**
TC94876	MT000689	CCR4-associated factor-like protein	7.42	1.0E-06	**7.51**
TC94955	MT000816	Probable WRKY transcription factor 41	2.38	2.2E-03	**2.81/10.31**
TC95121	MT007870	Isoprenylated protein FP6	3.30	4.9E-04	**1.87**
TC95260	MT000896	Respiratory burst oxidase homolog	2.33	1.3E-04	**2.04**
TC95378	MT009517	Serine/threonine protein kinase Nek2	8.41	4.2E-05	0.99
TC95389	MT007980	Drought-induced protein	2.62	4.9E-04	**1.54**
TC95994	MT009688	Receptor-like protein kinase	2.60	6.0E-06	**1.87**
TC96933	MT003663	DNA damage binding protein 1a	2.75	9.2E-05	1.00
TC97779	MT013317	F-box family protein-like	2.31	3.7E-05	**8.53**
TC99375	MT013180	Calmodulin-like protein	2.83	1.6E-04	**8.40**
TC99939	MT006001	Phosphate transporter 2	1.94	1.4E-04	**3.35/2.52**
TC100163	MT000865	PVPR3 protein	2.38	2.1E-03	**1.75/1.83**
TC100273	MT014378	Calcium-dependent protein kinase	2.07	1.8E-03	**2.46**
TC100307	MT001046	Ycf1 protein	3.81	9.2E-04	**2.81/5.94**
TC100528	MT001094	NAC-domain protein 3	2.66	1.1E-06	**4.84**
TC100577	MT007361	Putative DNA-binding protein	1.95	1.6E-04	**2.26**
TC100621	MT000985	Plastid-encoded RNA polymerase beta subunit	2.63	9.8E-05	**1.64/4.77**
TC100770	MT007670	Zinc finger-like protein	2.72	8.7E-06	**4.23**
TC100774	MT014422	Phi-1 protein	4.07	4.9E-05	**1.91**
TC100777	MT007470	SCOF-1	2.32	1.0E-04	**3.71/2.79**
TC100991	MT000719	AP2/EREBP transcription factor	3.04	3.7E-06	**2.41**
TC101262	MT000812	Syringolide-induced protein 13-1-1	9.72	2.6E-07	**9.27**
TC101396	MT008018	Ethylene-responsive element binding protein	2.62	1.4E-04	**2.03**
TC101633	MT015034	Protein transcription factor F2P9.5	3.00	7.0E-05	**4.64**
TC101742	MT009512	Transthyretin-like protein	1.88	2.7E-05	1.05
TC102042	MT008485	Calcium/calmodulin protein kinase 1	1.96	2.7E-03	**4.94**
TC103661	MT009901	Receptor protein kinase-like protein	2.45	1.5E-03	**1.69**
TC106220	MT015010	NBS-LRR-Toll resistance gene analog protein	2.51	2.1E-05	**1.57**
TC106735	MT015271	Ethylene-responsive element binding factor 5	2.08	1.5E-02	**2.07**
TC106805	MT007255	Homeodomain-leucine zipper protein 56	2.02	8.5E-05	**2.51**
TC106826	MT000306	Elicitor-inducible protein	1.95	8.4E-05	**3.83**
TC106943	MT014611	Probable glutathione-*S*-transferase	1.97	8.8E-04	**4.67**
TC107003	MT007472	Triacylglycerol lipase-like protein	3.18	6.7E-06	**3.43**
TC107019	MT007581	Transcription factor DRE-binding factor 2	2.07	3.2E-04	**2.78**
TC107302	MT007684	PDR1 ABC transporter	2.02	9.8E-05	**1.52**
TC107426	MT000934	Protein phosphatase 2C	3.90	4.4E-04	**3.11**
TC107549	MT008021	GmMYB29A2 protein	3.74	6.2E-05	**2.66/4.79**
TC107728	MT008702	Calcium-binding protein	2.22	3.7E-04	**2.08**
TC107974	MT008114	Avr9/Cf-9 rapidly elicited protein 31	2.96	1.7E-04	**2.40/3.28**
TC108715	MT009187	WRKY-type transcription factor	3.44	8.3E-04	**2.73**
TC108807	MT009854	At5g45300	3.18	7.1E-04	**2.74**
TC108912	MT003279	Dehydration-induced protein ERD15	3.04	1.1E-06	**3.29**
BQ141456	MT007083	1-aminocyclopropanecarboxylic acid oxidase	1.80	3.4E-03	1.17
Downregulated genes					
CA922447	MT007662	MTD1	0.52	2.8E-03	**0.30**
TC100156	MT007258	Narbonin	0.29	5.1E-03	**0.10/0.22**
TC100317	MT007071	Plastidic aldolase	0.61	3.1E-03	**0.56**
TC101996	MT002485	Pathogenesis-related protein 1	0.59	1.6E-02	**0.47**

Table 2 *Continued*

			Mt/Gi shoot versus Mt shoot		
TIGR ID	Oligo ID	Putative annotation	Array ratio	*P*-value	Quantitative RT-PCR ratio[a]
TC102760	MT003200	Nodulin homologous to narbonin	0.35	2.5E-03	**0.11/0.18**
TC103145	MT015279	MtN5 protein precursor	0.62	3.9E-03	**0.49/0.60**
TC106520	MT015174	Ubiquitin carboxyl-terminal hydrolase	0.46	6.0E-04	0.80
TC106522	MT015175	Early nodulin 12 precursor	0.43	2.2E-03	**0.43**
TC108388	MT002972	CPRD49 protein	0.45	1.5E-03	**0.41**
TC108642	MT010964	9-*cis*-epoxycarotenoid dioxygenase 4	0.47	1.5E-03	**0.66**
TC109886	MT015854	Cation-efflux transporter	0.59	1.8E-03	0.89
TC94063	MT007052	Auxin-induced β-glucosidase	0.57	4.8E-03	**0.46**
TC97434	MT013315	Ultraviolet-B-repressible protein	0.55	3.3E-03	**0.42**
TC97751	MT004918	DnaJ -like protein	0.42	3.4E-03	**0.26**/1.27
TC98393	MT004103	β-galactosidase	0.48	2.0E-03	**0.53**
TC99426	MT005039	RPT2	0.58	2.2E-03	**0.52**

[a]Quantitative RT-PCR ratios in bold are considered significant (>1.5-fold or <0.67-fold)
[b]The ratios are from two independent biological replicate experiments, one of which was used for the array hybridization.

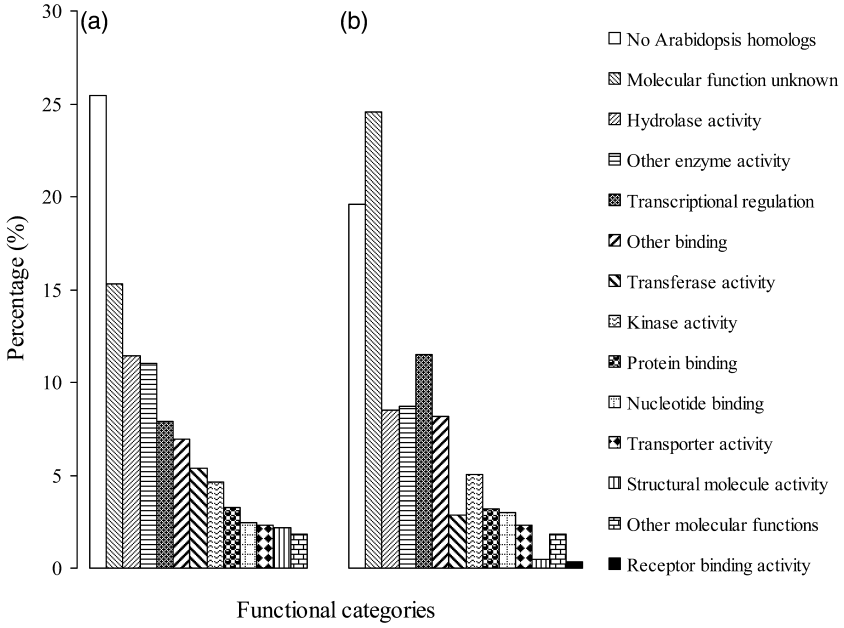

Figure 1. Molecular functional categories of genes showing differential expression in (a) the roots and (b) the shoots of *M. truncatula*/*G. intraradices* plants.
Molecular functions were obtained by comparison with the TAIR Slim Gene Ontology (GO) protein database (threshold of e-10).

Transcriptional responses of M. truncatula *roots to AM fungi from two genera*

M. truncatula has the capacity to form AM associations with a broad range of AM fungi, and, in some cases, the development and patterns of fungal growth within the roots vary. A second aim of this study was to determine whether there is a set of genes in *M. truncatula* that is regulated in the roots in response to different AM fungi. The *M. truncatula*/*Gigaspora gigantea* interaction differs from the *M. truncatula*/*G. intraradices* interaction in that *Gigaspora gigantea* shows a greater propensity for intra-cellular growth and creates coils in the outer cortical cells. Furthermore, fungi of the Gigasporaceae do not produce vesicles in the roots. In contrast, *G. intraradices* generally takes an inter-cellular route through the outer cortex and later shows extensive vesicle formation. In both symbioses, arbuscules are formed in the inner cortical cells.

M. truncatula seedlings were inoculated with *Gigaspora gigantea*, harvested at 28 dpi, and array experiments carried out with three biological replicates. In contrast to the earlier arrays, the data showed a greater level of variability, and consequently, the number of differentially expressed genes that passed the significance threshold was lower: 107 upregulated genes and 50 downregulated genes were identified in *M. truncatula*/*Gigaspora gigantea* roots (Table S4). Analysis of the overlap between the *M. truncatula*/*G. intraradices* and *M. truncatula*/*Gigaspora gigantea*

root data sets revealed 56 genes that were induced in both AM symbioses. The common denominator in these experiments was the AM symbiosis, and otherwise the plants differed in age and growth conditions. Consequently, we reasoned that the genes showing induction in both interactions might represent a core set of mycorrhiza-regulated genes. To further evaluate this gene set, expression of 41 of the induced genes was assessed in a third mycorrhizal association, *M. truncatula* colonized with *G. versiforme*. Thirty-nine of the 41 genes tested showed a greater than 1.5-fold increase in *M. truncatula/G. versiforme* roots (Table 3). As these genes are induced in AM symbioses with

Table 3 Expression of 41 genes in *M. truncatula* mycorrhizal roots during interactions with three AM fungi: *Glomus intraradices*, *Gigaspora gigantea* and *Glomus versiforme*

			Mt/Gi array data[a]		Mt/Gigi array data[b]		Mt/Gv quantitative RT-PCR[c]
TIGR ID	Oligo ID	Putative annotation	Ratio	*P* value	Ratio	*P* value	Ratio
TC94031	MT007390	No homology	3.09	3.1E-04	2.21	3.4E-07	**6.18**
TC94779	MT010042	Sorbitol dehydrogenase 8	2.35	3.1E-04	1.84	2.2E-02	**1.71**
TC94919	MT007813	β-tubulin	2.11	6.7E-03	1.53	2.8E-04	**6.83**
TC95776	MT014658	Annexin	1.42	1.1E-02	1.79	2.7E-06	1.13
TC95933	MT004574	AT5g01740	3.30	6.9E-05	2.54	2.7E-08	**3.55**
TC96102	MT013074	Odd Oz/10-m homolog 2	2.60	7.9E-03	1.82	3.4E-03	**55.47**
TC96159	MT010087	Hypothetical protein	13.36	1.1E-04	11.32	1.3E-05	**2.86**
TC96500	MT015668	Blue copper-binding protein-like	3.20	4.8E-04	3.24	7.2E-05	**270.66**
TC96500	MT015669	Blue copper-binding protein-like	4.57	7.1E-05	3.50	3.3E-05	**270.66**
TC96553	MT009937	Pectinesterase 3 precursor	15.35	7.3E-05	11.76	8.0E-07	0.97
TC97181	MT010084	Cytidine deaminase-like protein	3.96	5.2E-04	3.64	7.2E-05	**1.66**
TC98064	MT013394	Cysteine-rich anti-fungal protein 2	10.99	2.0E-06	5.80	6.9E-09	**861.04**
TC100115	MT013534	Glutathione-*S*-transferase GST 18	1.80	9.8E-03	1.60	3.3E-03	**2.13**
TC100720	MT009013	Glutathione-*S*-transferase-like protein	9.14	5.2E-04	10.37	9.8E-06	**31441.57**
TC100851	MT007526	Multifunctional aquaporin	5.33	1.1E-06	5.29	2.2E-08	**8.21**
TC100867	MT008641	No homology	5.82	9.6E-04	4.95	4.0E-06	**432.19**
TC101060	MT014645	Defensin AMP1	23.39	1.5E-03	7.01	4.4E-08	R[d]
TC101124	MT011611	Putative signal peptidase I	1.46	1.2E-02	1.44	7.5E-03	**3.08**
TC101627	MT001930	Transfactor-like protein	9.42	6.5E-06	9.41	3.4E-07	R
TC101698	MT004097	Allyl alcohol dehydrogenase	1.81	3.5E-03	2.10	5.0E-07	**2.64**
TC101749	MT013816	Bark lectin II precursor	5.94	5.4E-05	4.36	3.6E-06	**9959.87**
TC102074	MT012026	Isoflavonoid glucosyltransferase	3.35	3.2E-03	1.50	3.4E-02	**301.06**
TC102289	MT009234	BURP domain-containing protein	2.60	1.8E-05	1.62	1.1E-05	**2.59**
TC103034	MT010076	No homology	3.86	1.8E-06	3.92	3.0E-07	**1.99**
TC103212	MT009454	ent-kaurene oxidase	1.95	1.5E-05	1.45	1.1E-04	**2.88**
TC104204	MT004625	ent-kaurene synthase A	3.16	5.7E-05	1.48	1.1E-02	**13.93**
TC105073	MT013028	Pathogenesis-related protein PR-6	6.02	3.0E-05	5.64	1.5E-07	**10.65**
TC105217	MT012850	Subtilisin-like serine protease	2.08	6.8E-04	1.81	5.2E-05	**4.60**
TC105406	MT016058	Tubulin alpha chain (α-tubulin)	3.32	2.9E-03	1.72	6.6E-06	**6904.90**
TC105494	MT011939	Putative cytochrome P450	2.09	2.9E-02	2.54	2.6E-03	**1176.62**
TC106351	MT006798	α-amylase/subtilisin inhibitor	2.95	1.8E-04	2.00	1.2E-04	**10.27**
TC106954	MT009185	MtSCP1	6.12	8.7E-04	3.73	4.5E-06	**5573.97**
TC106955	MT009186	Serine carboxypepsidase	5.68	3.3E-04	3.85	9.6E-08	**6.13**
TC107070	MT003225	Blue copper-binding protein-like	2.92	8.0E-04	2.18	3.9E-05	**908.21**
TC107167	MT007595	SRG1-like protein	2.09	6.9E-05	1.55	2.6E-05	**8.34**
TC107197	MT002169	Specific tissue protein 2	7.49	3.2E-04	2.97	8.4E-06	**450.17**
TC107197	MT014644	Specific tissue protein 2	8.16	1.6E-05	2.11	1.2E-04	**450.17**
TC107460	MT007828	Ntdin	1.71	1.4E-03	1.81	1.4E-04	**3.68**
TC107461	MT015457	Ntdin	1.49	1.7E-02	1.47	2.7E-03	**3.68**
TC110146	MT010325	No homology	1.47	8.3E-03	1.58	1.1E-02	**11.31**
TC110699	MT013567	Nodulin	2.38	1.2E-03	1.46	6.3E-03	**5.90**
TC111056	MT010981	Nodulin	1.95	3.3E-02	1.58	2.7E-02	**101.64**
TC112474	MT015000	No homology	11.42	7.9E-05	1.61	2.4E-04	**248.35**

[a]Microarray ratio of expression in *M. truncatula/G. intraradices* (Mt/Gi) versus mock-inoculated *M. truncatula* roots.
[b]Microarray ratio of expression in *M. truncatula*/*Gigaspora gigantea* (Mt/Gigi) versus mock-inoculated *M. truncatula* roots.
[c]Quantitative RT-PCR ratio of expression in *M. truncatula*/*G. versiforme* (Mt/Gv) versus mock-inoculated *M. truncatula* roots. Ratios in bold are considered significant (>1.5-fold or <0.67-fold).
[d]Gene expression induced in *M. truncatula*/*G. versiforme* roots reported previously (Hanks *et al.*, 2005, Liu *et al.*, 2003).

three different fungal symbionts from two different genera and under variable growth conditions, they are good candidates for 'core' AM-induced genes. It should be noted that the age and growth conditions of the plants used in the two array experiments were not constant, therefore the *M. truncatula*/*G. intraradices* and *M. truncatula/Gigaspora gigantea* induced genes whose expression did not overlap, do not necessarily reflect unique differences between these two AM symbioses. Interestingly, one gene, TC104515, a cysteine-rich protein, showed a large increase in expression in *M. truncatula*/*G. intraradices* roots but was not induced in the symbiosis with *Gigaspora gigantea* or *G. versiforme*. The gene is not expressed in non-colonized roots, and showed a 6659-fold induction in *G. intraradices*-inoculated roots as assessed by quantitative RT-PCR. This is the only gene for which we could find strong evidence of induction in one AM symbiosis only.

Local and systemic transcriptional responses in mycorrhizal root systems

Mycorrhizal root systems are heterogeneous and are composed of both colonized and non-colonized roots that can be expected to show differing gene expression patterns. Through the use of promoter–reporter gene fusions, it is apparent that the expression of some mycorrhiza-induced genes is restricted to the colonized regions of the roots, while others are expressed systemically throughout the root system (Gianinazzi-Pearson *et al.*, 2000; Hohnjec *et al.*, 2005; Liu *et al.*, 2003; Maldonado-Mendoza *et al.*, 2005; Manthey *et al.*, 2004; Wulf *et al.*, 2003). To obtain an overview of the expression patterns of the mycorrhiza-induced genes from the core gene set, we used split-root experiments to monitor local versus systemic changes in gene expression in mycorrhizal roots. Two replicate experiments with *M. truncatula*/*G. intraradices* were established, and gene expression in the inoculated and mock-inoculated halves of the split-root system was measured relative to a split-root control experiment in which half of the root system was mock-inoculated. The expression of a *G. intraradices* α-tubulin gene (Corradi *et al.*, 2004) was monitored to demonstrate that the fungus was restricted to the inoculated half of the root system. The expression patterns of four *M. truncatula* genes (MtPT4, MtSCP1, blue copper-binding protein-like protein and bark lectin II precursor), whose expression patterns are known to be restricted to colonized roots, served as positive controls (Table 4; Frenzel *et al.*, 2005; Harrison *et al.*, 2002; Hohnjec *et al.*, 2005; Liu *et al.*, 2003). Using this approach, we identified 10 genes whose transcripts were highly induced in colonized roots and were undetectable in the other half of the root system (Table 4). This includes three cysteine-rich proteins, a protease inhibitor, a cytochrome P450 and an isoflavonoid glucosyltransferase. Three genes, TC111056, TC100875 and TC107460, showed elevated transcript levels in both the inoculated and non-inoculated halves of the root system, indicating that induction of these genes occurred systemically within the root system. Three genes, encoding a putative RING protein, a putative signal peptidase I protein and an unknown protein, showed elevated expression in inoculated root tissues and no change in the non-inoculated tissues. Finally a gene encoding a multifunctional aquaporin, was induced in the inoculated root tissues, but downregulated in the non-inoculated half of the root system. Thus, the split-root experiments reveal differential transcriptional responses in the mycorrhizal root system.

As indicated by the expression profiles of the shoots of the mycorrhizal plants, systemic transcriptional changes are also triggered in the shoots. We selected a subset of genes that were upregulated in the shoots and were also expressed in roots, and assayed their expression in split-root experiments (Table S5). This analysis identified an additional 23 genes whose expression is induced systemically in the mycorrhizal root system. Furthermore, three additional distinct expression patterns were observed, and the genes were grouped based on these patterns. Genes in group 1 showed a significant reduction in transcript levels in the colonized half of the root system but were induced in the non-colonized half of the root system. Genes in group 2 showed no significant alteration in the colonized half of the root system but a modest induction in the non-colonized half, and, in group 3, alterations in gene expression occurred only in the shoots of mycorrhizal plants. Overall, these data indicate that a complex set of transcriptional changes occurs locally and systemically throughout the mycorrhizal plant.

M. truncatula/G. intraradices *plants show elevated defense and stress response transcripts in the shoots and display enhanced resistance to* Xanthomonas campestris *pv.* alfalfae

Twenty-six percent of the genes induced in the shoots of the mycorrhizal plant are predicted to be involved in abiotic or biotic stress signalling or responses. This includes six genes whose predicted proteins share similarity with Avr9/Cf9 rapidly elicited proteins, seven genes annotated as ethylene-responsive element binding proteins, and five genes that share similarity with syringolide-induced genes, as well as numerous other genes associated with defense responses (Table S6). The levels of induction are modest but consistent. To determine whether this occurs in response to other AM fungi, a random selection of 10 defense/stress-related genes was monitored in the shoots of *M. truncatula/Gigaspora gigantea* and *M. truncatula/G. versiforme* mycorrhizal plants. Two biological replicates were evaluated, and the induction of the majority of these genes suggests that this response occurs following colonization with *Gigaspora*

Table 4 Twenty-one genes from the core mycorrhiza-induced gene set: expression in the colonized and non-colonized sections of a split-root system

			Quantitative RT-PCR expression ratio[a]			
			Experiment 1		Experiment 2	
TIGR ID	Oligo ID	Putative annotation	Gi[b]	Non-Gi	Gi	Non-Gi
TC105406	MT016058	α-tubulin[c]	**7694.88**	ND	**90 418.40**	ND
TC94453	MT009707	MtPT4	**9546.35**	ND	**18 390.65**	ND
TC106954	MT009185	MtSCP1	**143.63**	ND	**10 119.08**	ND
TC107070	MT003225	Blue copper-binding protein-like	**5395.24**	ND	*	*
TC101749	MT013816	Bark lectin II precursor	**21 882.07**	ND	*	*
TC101060	MT014645	Putative defensin AMP1 protein	**34 577.53**	ND	**3 339 405.20**	ND
TC112474	MT015000	No homology	**17 212.54**	ND	**461 354.90**	ND
TC100867	MT008641	No homology	**7900.25**	ND	**259 459.08**	ND
TC100804	MT015421	Predicted protein, partial	**2719.38**	ND	**52 852.77**	ND
TC104515[d]	MT006789	Cysteine-rich anti-fungal protein	**2228.44**	ND	**26 218.11**	ND
TC105494	MT011939	Putative cytochrome P450	**1348.06**	ND	**13 810.82**	ND
TC106351	MT006798	α-amylase/subtilisin inhibitor	**761.67**	ND	**6632.28**	ND
TC98064	MT013394	Cysteine-rich anti-fungal protein 2	**340.15**	ND	**5098.57**	ND
TC102074	MT012026	Isoflavonoid glucosyltransferase	**105.19**	ND	**1350.42**	ND
TC107197	MT002169	Specific tissue protein 2	**9288.00**	ND	N/A	N/A
TC111056	MT010981	Nodulin	**1.61**	**3.11**	**11.78**	**2.16**
TC100875	MT000486	Respiratory burst oxidase	**2.01**	**2.98**	**1.93**	**1.88**
TC107460	MT007828	Ntdin	**2.47**	1.24	**2.95**	**2.20**
TC96102	MT013074	Zinc finger, RING-type	**12.87**	0.92	**204.40**	**1.58**
TC101124	MT011611	Putative signal peptidase I	**1.96**	0.87	**14.08**	**0.51**
TC100812	MT002208	Hypothetical protein	**47.44**	0.77	N/A	N/A
TC100851	MT007526	Multifunctional aquaporin	**2.20**	**0.52**	**14.54**	**0.53**

[a]Quantitative RT-PCR expression ratios were obtained by comparing the Gi-colonized roots or the Gi-non-colonized roots versus a mock-inoculated split-root control. Ratios in bold are considered significant (>1.5-fold or <0.67-fold). ND, no transcripts detected; *promoter localization results of these genes have shown a similar localized expression pattern; N/A, data not available.

[b]Gi and non-Gi indicate the colonized half and the non-colonized half of the split-root experiment. Experiments 1 and 2 are two independent split-root experiments.

[c]Expression of the α-tubulin gene was monitored to demonstrate that the non-colonized half of the split-root experiment was free of *G. intraradices*.

[d]TC104515 was induced only in *M. truncatula* roots colonized with *G. intraradices*.

gigantea and with *Glomus versiforme* (Table S7). In some cases, the AM symbiosis has a bioprotective effect against root pathogens (Cordier *et al.*, 1998); however, the effects on shoot pathogens are less clear and have not received significant attention. Systemic resistance to leaf pathogens induced by root bacteria is well known and has been dissected extensively in Arabidopsis (Cartieaux *et al.*, 2003; Pieterse *et al.*, 1996; Timmusk and Wagner, 1999). The induction of stress and defense-related genes in the shoots of mycorrhizal plants prompted us to test the hypothesis that development of the mycorrhizal symbiosis increases the resistance of the plant to subsequent challenge by virulent leaf pathogens. We selected a bacterial pathogen, *Xanthomonas campestris* pv. *alfalfae*, that infects *M. truncatula* A17 (Gamas *et al.*, 1998), and challenged mycorrhizal and non-mycorrhizal control *M. truncatula* plants with 10^5 CFU ml^{-1} *Xanthomonas campestris* pv. *alfalfae.* At 3 days post-infiltration, the bacterium population in the leaves of mycorrhizal plants was $1.2 \pm 0.7 \times 10^4$ CFU cm^{-2} leaf area, 4.4-fold less than the corresponding non-mycorrhizal control plants ($5.2 \pm 1.0 \times 10^4$ CFU cm^{-2} leaf area) (Figure 2). The difference was greater at 6 days post-infiltration, and mycorrhizal plants showed populations that were eightfold lower than the respective controls. In addition, at 6 days post-infiltration, a visible difference in the leaves became apparent, and the non-mycorrhizal controls showed slightly more yellowing relative to the leaves of the mycorrhizal plants (Figure S2a,b). At 9 days post-infiltration, the bacterium population in the leaves of non-mycorrhizal control plants reached $12.1 \pm 5.5 \times 10^6$ CFU cm^{-2} leaf area, with severe disease symptoms including some necrotic leaves, while the bacterium population in the leaves of mycorrhizal plants was only $3.3 \pm 1.0 \times 10^6$ CFU cm^{-2} leaf area and the disease symptoms were much less severe (Figure S2c,d). The differences in bacterium populations in the mycorrhizal and non-mycorrhizal plants were significant at all three time points ($P < 0.05$). The transcriptional profile of the mycorrhizal shoots and the significant reduction in *Xanthomonas campestris* pv. *alfalfae* growth in the mycorrhizal plants supports the hypothesis that the AM symbiosis results in a

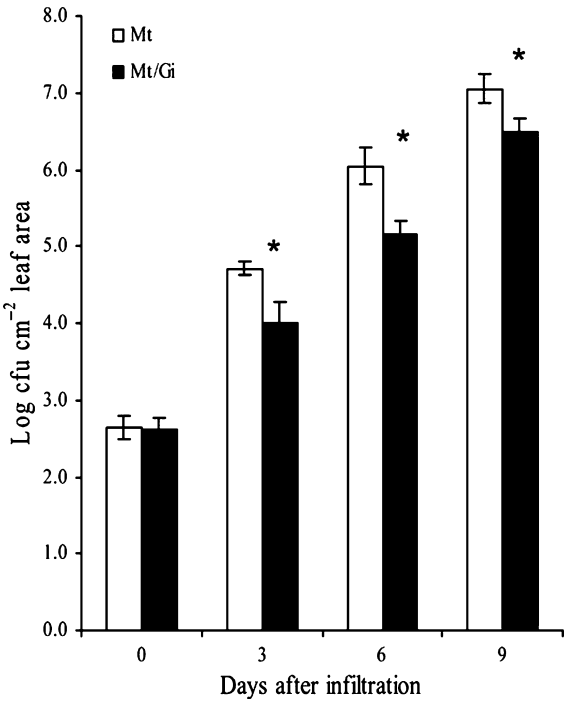

Figure 2. *Xanthomonas campestris* pv. *alfalfae* growth in the leaves of *M. truncatula* mycorrhizal plants (*M. truncatula/G. intraradices*, (Mt/Gi) or mock-inoculated (Mt) plants.
Data presented are the number of colony-forming units (CFU) per cm^2 of leaf tissue sampled at 0–9 days post-infiltration. Error bars represent the SD ($n \geq 8$). Asterisks indicate a significant difference between treatments ($P < 0.05$). Two independent experiments were performed with similar results. The data shown are the results of one of two independent experiments.

systemic increase in basal defenses that functions to enhance resistance to subsequent challenge by a bacterial pathogen.

Discussion

It is well established that the interaction of microbes with plants can induce both local and systemic alterations in the plant (Bostock, 2005; Glazebrook, 2005). Here, transcriptional profiling and quantitative RT-PCR analyses revealed local and systemic changes in gene expression in roots, as well as systemic alterations in the transcriptome of the shoots of *M. truncatula* in response to colonization by AM fungi. Using the *M. truncatula* 16 K oligonucleotide array, we identified a set of 647 genes differentially expressed in *M. truncatula*/ *G. intraradices* roots. Of these, 161 have been reported recently by Hohnjec *et al.* (2005), but the remaining 486 are mostly novel. The study by Hohnjec *et al.* utilized the same *M. truncatula* 16 K oligo set and included a comparison of gene expression under high- and low-P_i growth conditions. We had designed our experiment to avoid alterations associated with P_i nutrition, and a comparison of the 647 genes identified from our array experiment with the Hohnjec P_i-related dataset indicates that our strategy was largely successful. Only 10 *M. truncatula* genes, seven upregulated (TC94543, TC109049, TC100841, TC106351, TC107918, TC107197, TC100386) and three downregulated (TC100658, TC94274, BI270295), are regulated in response to high-P_i conditions. This includes Mt4 (TC100658), a P_i starvation-induced gene whose expression is downregulated by high P_i and also in the AM symbiosis (Burleigh and Harrison, 1998). Consequently, we conclude that the majority of the transcriptional changes observed on our array are a consequence of fungal colonization of the root system rather than the result of enhanced P_i nutrition.

Most of the *M. truncatula* microarray profiling experiments reported to date have explored the transcriptional responses of roots to colonization by *Glomus* species including *G. intraradices*, *G. mosseae* and *G. versiforme* (reviewed by Hohnjec *et al.*, 2006). Currently, the AM fungi are classified into four families with 10 genera (Schwarzott *et al.*, 2001), and while they are considered non-specific with respect to host range, there is variation in their growth patterns within the roots and in functionality (Smith *et al.*, 2004). To determine whether there are similarities in the transcriptional response of *M. truncatula* to fungi from different genera, we profiled gene expression in *M. truncatula* roots colonized with *Gigaspora gigantea*, a member of the Gigasporaceae (Walker and Schussler, 2004; Walker *et al.*, 2004), and compared this with the *M. truncatula*/*G. intraradices* array data. Fifty-six genes were induced in both symbioses, indicating similarities in the *M. truncatula* response to these fungi. Thirty-nine genes from this set were induced in *M. truncatula* roots colonized with *G. versiforme* also. Although labelled as a '*Glomus*', *G. versiforme* has recently been reclassified into the Diversisporaceae within the Diversisporales and is distinct from the Glomales family, of which *G. intraradices* is a member (Walker and Schussler, 2004; Walker *et al.*, 2004). The transcript data indicate that, despite apparent differences in the morphology of the mycorrhizal associations, a component of the transcriptional response to the *G. intraradices*, *G. versiforme* and *Gigaspora gigantea* is identical. Thirty-four genes from this core set of 56 genes are also induced in *M. truncatula*/*G. mosseae* mycorrhizal roots, further supporting their designation as a core set of genes induced in response to a broad variety of AM fungi (Hohnjec *et al.*, 2005). In rice, comparative transcriptome analyses identified an overlap in the response to mycorrhizal fungi and fungal pathogens (Guimil *et al.*, 2005). While some of the responses were defense-associated, most were not, and pointed to common compatibility responses to microbes with diverse lifestyles. It will be interesting to determine the extent to which our 'core' set of mycorrhiza-induced genes is utilized in other interactions. Our current core mycorrhiza-induced gene set contains 56 genes and this is certainly incomplete. Biological and technical vari-

ation arising in the array experiments resulted in the loss of data, particularly those genes whose transcript levels were low. Quantitative RT-PCR analysis of genes predicted from the array data to be induced only in the *M. truncatula*/*G. intraradices* interaction revealed that they were induced in the *M. truncatula/G. gigantea* and *M. truncatula/G. versiforme* interactions also. Based on these analyses, we predict that the core mycorrhiza-induced gene set will be large, and gene induction specific to a single AM symbiosis will be rare. Following extensive analysis, there was only one *M. truncatula* gene (TC104515) for which we could obtain solid evidence of an induction response specific to one AM fungus. TC104515 is predicted to encode a cysteine-rich protein, and transcripts were detected only in roots colonized with *G. intraradices* (6659-fold induction) and not in roots colonized with *G. versiforme* or *Gigaspora gigantea*. The data were confirmed in multiple biological samples. This gene was not expressed in *M. truncatula*/*G. mosseae* roots (Hohnjec *et al.*, 2005), and, in the TIGR Medicago gene index, it is represented by ESTs exclusively from *M. truncatula*/*G. intraradices* cDNA libraries. The identity of the gene is somewhat surprising, as a broad array of small, cysteine-rich proteins are induced in AM roots. Among the genes induced in the three AM symbioses there are two other small cysteine-rich proteins (TC101060 and TC98064) that share 84% identity in the so-called 'defensin domain'. The *G. intraradices*-specific cysteine-rich protein shares only 48% identity with the defensin domain of TC101060. Data from the split-root experiments reveal that expression of these cysteine-rich proteins occurs only in the colonized regions of the root system. These proteins have been named defensins because the original members of the family displayed anti-fungal activity (Terras *et al.*, 1995). However, it is becoming clear that some of them have roles other than defense, and they have been shown to have an effect on heavy metal stress (Mirouze *et al.*, 2006) and to modulate the activity of calcium channels (Spelbrink *et al.*, 2004). Calcium is implicated as a signal in the AM symbiosis (Ivashuta *et al.*, 2005; Levy *et al.*, 2004; Mitra *et al.*, 2004), and it is possible that the roles of the cysteine-rich proteins in mycorrhizal roots relate to this.

The split-root experiments indicated that 10 genes from the core mycorrhiza-induced gene set show expression locally in the roots in response to the symbiosis, and transcripts were not detectable in the absence of the fungal symbiont. Among these, the activation of cytochrome P450 and isoflavonoid glucosyltransferase in the colonized tissues is consistent with earlier findings that phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS) transcripts were elevated specifically in the cells with arbuscules (Harrison and Dixon, 1994). Together, these data suggest that secondary metabolite biosynthesis is activated locally in the vicinity of the fungus. Two protease inhibitors, one member of the Kunitz inhibitor class and one member of the α-amylase/subtilisin class, are highly induced in the colonized regions of the root system. Proteases predicted to be inhibited by these classes of inhibitors show induction in mycorrhizal roots, and expression of one of these, a putative serine carboxypeptidase, occurs exclusively in colonized roots (Table 4; Liu *et al.*, 2003).

In addition to the identification of genes showing induction in the vicinity of the fungus, the split-root experiments uncovered a complex array of expression patterns within the whole mycorrhizal root system. This includes genes induced in both colonized and non-colonized sections of the root system, and genes induced in colonized roots but downregulated in non-colonized roots. The former group includes a nodulin (TC111056), a respiratory burst oxidase (TC100875) and Ntdin (TC107460), a chloroplast protein involved in the synthesis of molybdenum co-factor (Yang *et al.*, 2003). The latter group includes a gene predicted to encode a multifunctional aquaporin (TC100851). Furthermore, comparison of the two replicate experiments reveals an interesting difference in the response in the colonized versus non-colonized sections of the root system. Based on the expression level of the marker genes MtPT4 and SCP1, the roots in experiment 2 show higher levels of fungal colonization than those of experiment 1 (Table 4). Accordingly, most, but not all, of the genes showing systemic transcriptional responses have higher transcript levels in the colonized roots of experiment 2 relative to the colonized roots of experiment 1. Consequently, it is intriguing that, for each gene, the fold-change in transcripts in the non-colonized sections of the root systems is similar in both experiments. This suggests that the transcriptional response in the non-colonized section of the root system is not influenced by the level of colonization of the other half of the root system, but instead occurs at a certain level as a consequence of the presence of the fungus. For those genes that show an increase in transcript levels, such expression patterns might occur if the gene shows a limited response to a systemic signal but is also activated in a spatially restricted manner in cells colonized by the fungus. However, this model cannot be invoked for the aquaporin, where the transcriptional responses in the colonized and non-colonized sections of the root system occur in opposite directions. These complex gene expression patterns also provide an explanation for variation in gene expression levels reported for some mycorrhiza-regulated genes. If a gene is regulated in an opposite manner in the colonized and non-colonized regions of the root, its overall transcript levels will be extremely sensitive to differences in the proportion of colonized and non-colonized roots in the root system, even more so than genes induced locally in the vicinity of the fungus.

By contrast with gene expression in mycorrhizal roots, the alterations in transcript levels in the shoots were mostly modest, in the order of two- to ninefold. All genes induced in the shoots showed basal expression in the shoots of

non-colonized plants, and thus mycorrhiza-specific shoot gene expression was not observed. The most highly upregulated genes share similarity with a U-box protein (TC101262, annotated as similar to syringolide-induced protein 13-1-1) and an F-box family protein (TC97779), suggesting a potential increase in ubiquitin-mediated protein degradation (Zeng *et al.*, 2006). A calmodulin-like protein (TC99375) and a calcium-dependent protein kinase (TC102042) were also upregulated eight- and fourfold, respectively, and suggest that calcium signalling occurs in the shoots as well as the roots of mycorrhizal plants. If these genes are not responsive to other stimuli, they may serve as useful shoot molecular markers of the AM symbiosis. Analysis of EST distribution suggests that the U-box protein gene is expressed in a variety of plant–microbe interactions, and therefore would not be a useful marker. In general, the proportion of induced genes involved in transcriptional regulation or signalling was greater in the shoots than in the roots. Further examination revealed that many of the genes showing differential expression in shoots shared similarities with defense or stress response genes or defense/stress signalling genes. In this group there were striking similarities to eight genes designated ACRE genes. These were reported previously as rapidly induced in tomato in response to the fungal pathogen *Cladosporium fulvum*, and suggested to be involved in the initial development of defense signalling (Durrant *et al.*, 2000). The group includes two glycosyl transferases, a calcium-binding protein, a kinase and the putative ubiquitin-protein ligase mentioned earlier. Based on the split-root analyses, some of these genes, including two WRKY-type transcription factors and a TOLL-type protein, were induced systemically in the roots, and showed a greater increase in transcripts in the non-colonized roots and shoots of the mycorrhizal plants. There was also a notable overlap with a group of soybean genes identified as 'rapidly induced in response to syringolide', a bacterial elicitor produced by *Pseudomonas syringae* (Hagihara *et al.*, 2004). In the original report, the putative functions of many of these genes were unknown and they were annotated simply as syringolide-induced protein. Current database searches revealed their identities, and they include two glycosyl hydrolases, two proteins involved in ubiquitin-mediated degradation and a xyloglucan endotransglycosylase. Again these genes were not only induced in the shoots of mycorrhizal plants, but subsequent analysis revealed that they were induced locally and systemically in the roots also.

The induction of a significant number of defense-associated transcripts suggested that development of the AM symbiosis might be inducing a response similar to the ISR response induced by rhizobacterium *Pseudomonas thivervalensis* (Cartieaux *et al.*, 2003). Consequently, we undertook functional experiments to test the hypothesis that development of a mycorrhizal symbiosis results in enhanced resistance to subsequent challenge with a leaf pathogen. In line with the ISR model, we selected a pathogen, *Xanthomonas campestris* pv. *alfalfae*, that is virulent on *M. truncatula*, and evaluated its growth rate in the leaves of non-mycorrhizal and mycorrhizal plants. Consistent with the transcriptional profile, the level of *X. campestris* growth attained in the non-mycorrhizal plants was significantly higher than that attained in the mycorrhizal plants, suggesting that the defense response predicted from the expression profiling is functional. The level of reduction in growth is similar to that observed in Arabidopsis following the induction of an ISR response. In Arabidopsis, transcriptional analysis of the ISR response identified 42 induced genes of which 10 were implicated in defense responses or disease resistance (Cartieaux *et al.*, 2003). In addition, photosynthesis-associated gene expression was downregulated in Arabidopsis leaves that were mounting an ISR response (Cartieaux *et al.*, 2003). In addition to parallels between ISR and mycorrhiza in the induction of basal defenses, the most highly downregulated gene in the shoots of mycorrhizal plants was the small subunit of Rubisco (Table S2). The mycorrhizal symbiosis has been associated with increased resistance to a root oomycete pathogen (Cordier *et al.*, 1998), but a significant resistance response in the shoots has not been reported previously. Here, the transcriptional data coupled with the functional data support the hypothesis that development of the AM symbiosis leads to a systemic increase in the basal disease resistance of the plant.

In summary, the AM symbiosis is accompanied by complex alterations in gene expression in the roots and shoots of mycorrhizal plants. This includes not only local and systemic changes in gene expression, but, in some instances, contrasting changes in expression in the colonized and non-colonized sections of the root. The root-specific responses, particularly those occurring locally in the vicinity of the fungus, are largely tuned to development of the interfaces required for the symbiosis, while a small but significant systemic increase in stress and defense response transcripts in the shoots is correlated with increased resistance to subsequent pathogen attack.

Experimental procedures

Plant materials, growth conditions and inoculation methods

Medicago truncatula cv. Jemalong line A17 plants were grown in growth rooms under a 16 h light (25°C)/8 h dark (22°C) regime. *G. intraradices* was maintained in cultured carrot roots (*Daucus carota*) on plates, and spores were prepared from the plates according to the method described by St-Arnaud *et al.* (1996). *Gigaspora gigantea* was maintained on Bahia grass (*paspalum notatum*), and spores were surface-sterilized according to the procedures described by Bécard and Fortin (1988). Stock cultures of *G. versiforme* was maintained on leek (*allium porum*) plants in pot culture, and the preparation of surface-sterilized spores has been

described previously (Harrison and Dixon, 1993; Liu *et al.*, 2003, 2004).

Fourteen-day-old *M. truncatula* plants were transplanted in 11 cm pots (eight plants per pot) and inoculated with 8000 *G. intraradices* spores per pot and fertilized twice weekly with half-strength Hoagland's solution containing 20 μM potassium phosphate. Plants were harvested at 30 or 28 days post-inoculation with *G. intraradices* or *G. versiforme*, respectively. The shoots and roots of the eight plants from each pot were pooled, and the fresh weights were measured (Table 1). Random root samples were collected for assessment of their colonization levels using the gridline intersect method (McGonigle *et al.*, 1990), and the rest was immediately frozen in liquid N_2 for further analysis. The three *G. intraradices*-inoculated replicates used in the array experiment were 49, 50% and 53% root length colonized (RLC; Table 1), and the fourth replicate used in the quantitative RT-PCR analysis was 48% RLC. The colonization level of the *G. versiforme*-inoculated roots used in the quantitative RT-PCR analysis was 44%.

For the inoculation experiments with *Gigaspora gigantea*, 3-day-old seedlings were transplanted into small cones (3 × 15 cm), 10 seedlings per cone, and inoculated with 50 spores. Fertilization was as described above. The plants were harvested at 28 dpi, and the colonization levels of the three replicates used in the array experiments were 39%, 47% and 44% (Table 1).

The expression of three marker genes was evaluated in two biological replicate samples each of *M. truncatula/G. intraradices*, *M. truncatula/G. versiforme* and *M. truncatula/Gigaspora gigantea* roots. Expression of the fungal α-tubulin gene provides an indication of the total amount of fungal biomass in the sample (including intraradical and extra-radical mycelium and spores). The expression of MtPT4 (Harrison *et al.*, 2002) and the blue copper-binding protein gene (Hohnjec *et al.*, 2005) provide an indication of the intensity of colonization within the roots. These data provide a useful complement to estimates of colonization based on RLC (Table S8).

For the split-root experiment, the root system of a 17-day-old *M. truncatula* plant was divided into two parts and maintained in two separate cones held together with rubber bands. Roots in cone one were inoculated with 1500 *G. intraradices* spores, and those in cone two were inoculated with water. A parallel control consisted of a split-root system where cone one was mock-inoculated with the final distilled-water wash from the spore purification procedure and cone two was inoculated with water. Two independent biological replicates were prepared and the roots were harvested at 22 dpi. The colonization levels of the inoculated root tissues in the two biological replicates were 29% and 21% RLC, respectively. Expression of the *G. intraradices* α-tubulin gene (Table 4) was monitored to confirm that the mock-inoculated control halves of the split-root system did not contain *G. intraradices*.

Total phosphate measurement

The total phosphate content of the leaves from the shoot materials used for the array experiment was measured by phosphomolybdate colorimetric assay as described previously (Ames, 1966).

RNA isolation, dye labelling of cDNA probes, and microarray hybridization

Total RNA was isolated with Trizol® reagent (Invitrogen; http://www.invitrogen.com/) with extra phenol–chloroform and chloroform purification steps, treated with RNase-free DNase (Promega; http://www.promega.com/) at 37°C for 20 min, and then purified using the RNeasy plant mini kit (Qiagen; www.qiagen.com/) according to the manufacturer's instructions. The yield and purity of the total RNA were quantified spectrophotometrically, and the integrity of the total RNA was examined on agarose gels.

Total RNA (25 μg) was used to generate fluorescently labelled cDNA probes using the SuperScript™ indirect cDNA labelling system (Invitrogen). For each experiment, three biological replicates were performed, and, to avoid potential variations in labelling efficiency of the fluorescent dyes, we also performed 'dye swap' experiments. Consequently, Cy3™- and Cy5™-labelled probes (Amersham Biosciences; http://www5.amershambiosciences.com/) were generated from each RNA sample. The cDNA yield and the labelling efficiency were assessed spectrophotometrically.

The Medicago Genome Oligo Set version 1.0 microarray (Qiagen Operon (http://omad.operon.com)) contains 16 086 70-mer probes designed according to TIGR Medicago Gene Index 5 (http://www.tigr.org/tdb/mtgi). Each 70-mer oligonucleotide corresponds to a unique *M. truncatula* TC (tentative consensus) sequence. Details of this oligo set are available at http://oligos.qiagen.com/arrays/omad.php. The probes were printed at the University of Arizona (http://www.ag.arizona.edu/microarray) on aminosilane-coated 'Superamine' slides (Telechem International, CA, USA (http://www.arrayit.com)) in a 12 meta-row × 4 meta-column configuration. The sub-array grid contains 23 × 23 spots. The diameter of each spot was approximately 100–110 μM and the spacing between spots was 185 × 185 μM. Slides were baked for 1 h at 80°C after printing and stored in a vacuum-sealed box at room temperature.

Before hybridization, the slides were re-hydrated over water at 50°C, and snap-dried on a 65°C heating block. The rehydration and drying were carried out four times, and then the slides were UV cross-linked at 360 mJ using a Stratalinker (Stratagene; http://www.stratagene.com/). The slides were then pre-hybridized for 2 h at 42°C in pre-hybridization solution (5x SSC, 0.1% SDS, 1% BSA).

Equivalent quantities of Cy3- and Cy5-labelled cDNA probes were combined and evaporated to dryness in a Speedvac (Savant, http://www.gmi-inc.com/products/savant%20speed%20vac.htm) in the dark. The combined dried cDNA probes were then suspended in 58 μl of hybridization solution containing 46 μl of 1.25x formamide-based Hyblt2™ hybridization solution (Telechem International), 1 μl poly(dA) and 11 μl H_2O. The resuspended cDNA probes were then incubated at 95°C for 5 min and applied to a pre-warmed (50°C) slide, and covered with a clean glass LifterSlip (Erie Scientific, http://www.eriemicroarray.com). The slides were then sealed in Corning hybridization chambers (Corning Inc., http://www.corning.com/lifesciences) and maintained at 43°C in a waterbath with gentle shaking at 40 rpm for 16–20 h in the dark. Four successive post-hybridization washes were conducted in Coplin jars (Fisher Scientific, http://www.fishersci.com), and comprised a wash in 1x SSC, 0.2% SDS at 43°C for 8 min, a wash in 0.2x SSC, 0.2% SDS at room temperature (RT) for 8 min, a wash in 0.1x SSC at RT for 8 min and finally a wash in 0.05x SSC at RT for 8 min, with gentle agitation during each wash. The slides were dried immediately via gentle centrifugation (150 g for 2 min) and stored in the dark until scanning.

For each array experiment, six slides were hybridized with RNA from three independent biological replicate samples, each with dye-swap labelling.

Data acquisition and data analysis

Slides were scanned using a two-channel confocal microarray scanner (ScanArray5000, GSI Lumonics, http://www.gsilumonics.com) and the associated ScanArray software (version 3.1, Packard BioChip Technologies, http://lasperkinelmer.com). Scans were conducted at a resolution of 10 μm, the laser power was set at 90%

of maximum, and the photo-multiplier tube (PMT) gain setting was between 68% and 85% of maximum based on the signal intensity of each slide. Fluorescence data were processed using ImaGene software (version 5.6; BioDiscovery Inc., http://biodiscovery.com) using default quality controls and segmentation values with appropriate adjustment according to the signal intensity of each slide.

Normalization and analysis of microarray data were performed using Genespring® software (version 6.2; Silicon Genetics, http://www.chem.agilent.com). For each experiment, data from six slides were imported into Genespring® software, and values for all spots on the arrays were normalized using per spot and per chip intensity-dependent (Lowess) normalization, using 20% of the data to calculate the Lowess fit at each point (Yang *et al.*, 2002). For the experiment comparing gene expression ratios in root tissues of *M. truncatula* versus *M. truncatula*/*G. intraradices* and *M. truncatula* versus *M. truncatula*/*Gigaspora gigantea*, significantly up- or downregulated genes were filtered for a *t* test *P* value < 0.05, and normalized expression ratios greater or less than 1.4- or 0.71-fold, respectively. Higher stringency conditions were applied for the experiment comparing gene expression ratios in *M. truncatula* versus *M. truncatula*/*G. intraradices* shoots, where significantly up- or downregulated genes were filtered for a *t* test *P* value < 0.05, and normalized expression ratios greater or less than 1.5- or 0.67-fold, respectively.

Real-time quantitative RT-PCR analysis

First-strand cDNA template was synthesized using SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The cDNA yield and concentration of final working solutions was measured and adjusted accordingly using an ND-1000 spectrophotometer (NanoDrop Technologies, http://www.nanodrop.com). Quantitative RT-PCR primers were designed using GenScript's online real-time PCR primer design tool (http://www.genscript.com/ssl-bin/app/primer). Primers were selected from every candidate TC sequence within or close to the unique oligomer probes (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=medicago). Oligonucleotide sequences of all the primers are listed in Table S9. Quantitative RT-PCR was carried out in optical 384-well plates and labelled with the SYBR Green PCR master mix (Applied Biosystems, http://www.appliedbiosystems.com) using the ABI PRISM 7900 HT sequence detection system (Applied Biosystems). Three technical replicates were conducted for each gene in a total volume of 15 μl containing 7.5 μl 2x SYBR Green PCR master mix, 200 nM of each primer, and 20 ng of cDNA template with the following PCR regime: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 30 sec, 56°C for 30 sec and 72°C for 30 sec. Melting curve analysis was performed after each reaction to exclude primers generating non-specific PCR products with the thermal cycle of 95°C for 15 sec, 60°C for 15 sec and 95°C for 15 sec. The generated data were analysed with SDS 2.2 software (Applied Biosystems). The optimal baseline range and threshold values were calculated using the automatic CT function available with the SDS 2.2 software. Two constitutively expressed *M. truncatula* genes that encode elongation factor 1-alpha (EF-1α) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were included in each PCR run for normalization of expression levels of the gene of interest (Liu *et al.*, 2003; Weidmann *et al.*, 2004; Wulf *et al.*, 2003). The amplification efficiencies (*E*) for EF-1α, GAPDH and eight randomly selected representative genes were determined according to the equation: $E = 10^{-1/\text{slope}}$ (Pfaffl, 2001), and all were >1.75. The calibration curves were created using a series of input cDNA concentrations from 0.3 to 30 ng, and were found to be highly linear (Pearson correlation coefficient $r > 0.98$).

Bacterial infection and measurement

Xanthomonas campestris pv. *alfalfae* (kindly provided by Professor Steven Beer, Department of Pathology, Cornell University, Ithaca, NY, USA) was grown on LB agar plates and resuspended in 10 mM $MgCl_2$ solution. The bacteria were then washed three times with 10 mM $MgCl_2$ and resuspended at a final concentration of 10^5 CFU ml^{-1}. Using a hypodermic syringe, the bacterial suspension was infiltrated into trifoliate leaves of *M. truncatula*/*G. intraradices* mycorrhizal plants (4 weeks post-inoculation with 1500 spores per plant) or *M. truncatula* mock-inoculated controls. Four or five trifoliate leaves were infiltrated per plant on each of eight mycorrhizal plants and 10 mock-inoculated plants. At days 0, 3, 6 and 9 after bacterial infiltration, a trifoliate leaf was removed from each plant, and three leaf discs from each infiltrated trifoliate leaf were sampled using a cork borer and macerated in 10 mM $MgCl_2$ containing 0.02% Silwet L-77 (OSi specialities, http://www.specialchem4coatings.com). Appropriate dilutions of the bacterial solutions were plated out, and colony-forming units (CFU) were counted. Thus at each time point, 10 trifoliate leaves were sampled from the mycorrhizal mock-inoculated plants and eight trifoliate leaves were sampled from the mycorrhizal plants. Following the harvest of the final leaves, root samples were then taken to assess the colonization level of the plants by *G. intraradices*. The 10 mock-inoculated plants did not contain any AM fungus.

The colonization levels of the eight *G. intraradices*-colonized plants ranged from 41% to 58% RLC as assessed by the modified gridline intersect method.

The bacterium populations in the mycorrhizal and mock-inoculated plants were analysed by analysis of variance (ANOVA) using the SAS program, version 9.1 (SAS Institute Inc., http://www.sas.com). When a statistically significant interaction ($P < 0.05$) was observed, differences in least square means were tested.

The data discussed in this publication have been deposited in the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) and are accessible at GEO Series accession number GSE5477.

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Supplementary material

The following supplementary material is available for this article online:

Table S1. Genes showing significant differential expression in *M. truncatula*/*G. intraradices* roots.

Table S2. Genes showing significant differential expression in *M. truncatula*/*G. intraradices* shoots.

Table S3. List of *M. truncatula* genes responsive to *G. intraradices* that have no Arabidopsis homolog, but have a rice ortholog.

Table S4. Genes showing significant differential expression in *M. truncatula*/*Gigaspora gigantea* roots.

Table S5. 44 genes differentially regulated in the shoots of mycorrhizal plants: expression in the colonized and non-colonized sections of a split root system.

Table S6. Genes showing significant differential expression in *M. truncatula*/*G. intraradices* shoots predicted to be involved in abiotic or biotic stress responses.

Table S7. qRT-PCR expression ratios of 10 defence-related genes in shoot tissues of plants inoculated with three different AM fungi.

Table S8. qRT-PCR expression ratios of *G. intraradices* alpha-tubulin gene and two mycorrhiza-induced plant genes in mycorrhizal root tissues.

Table S9. Primer sequences used in the real-time quantitative RT-PCR experiments.

Figure S1. Scatter plots of the expression ratios of genes in the root (a) and shoot (b) of *M. truncatula /G. intraradices* mycorrhizal plants.

Figure S2. Detached *M. truncatula* leaves six days (a and b) and nine days (c and d) after infiltration with *Xanthomonas campestris* pv. a*lfalfae*.

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

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