

Transcript Profiling Coupled with Spatial Expression Analyses Reveals Genes Involved in Distinct Developmental Stages of an Arbuscular Mycorrhizal Symbiosis^W

Jinyuan Liu,^a Laura A. Blaylock,^a Gabriella Endre,^b Jennifer Cho,^c Christopher D. Town,^c Kathryn A. VandenBosch,^b and Maria J. Harrison^{a,1}

^a Boyce Thompson Institute for Plant Research, Ithaca, New York 14853

^b Department of Plant Biology, University of Minnesota, St. Paul, Minnesota 55108

^c The Institute for Genomic Research, Rockville, Maryland 20850

The formation of symbiotic associations with arbuscular mycorrhizal (AM) fungi is a phenomenon common to the majority of vascular flowering plants. Here, we used cDNA arrays to examine transcript profiles in *Medicago truncatula* roots during the development of an AM symbiosis with *Glomus versiforme* and during growth under differing phosphorus nutrient regimes. Three percent of the genes examined showed significant changes in transcript levels during the development of the symbiosis. Most genes showing increased transcript levels in mycorrhizal roots showed no changes in response to high phosphorus, suggesting that alterations in transcript levels during symbiosis were a consequence of the AM fungus rather than a secondary effect of improved phosphorus nutrition. Among the mycorrhiza-induced genes, two distinct temporal expression patterns were evident. Members of one group showed an increase in transcripts during the initial period of contact between the symbionts and a subsequent decrease as the symbiosis developed. Defense- and stress-response genes were a significant component of this group. Genes in the second group showed a sustained increase in transcript levels that correlated with the colonization of the root system. The latter group contained a significant proportion of new genes similar to components of signal transduction pathways, suggesting that novel signaling pathways are activated during the development of the symbiosis. Analysis of the spatial expression patterns of two mycorrhiza-induced genes revealed distinct expression patterns consistent with the hypothesis that gene expression in mycorrhizal roots is signaled by both cell-autonomous and cell-nonautonomous signals.

INTRODUCTION

In natural ecosystems, the roots of most plants are actually symbiotic organs called mycorrhizae, a term with Greek origins that literally means “fungus root.” There are many types of mycorrhizal symbioses, the most common of which is the arbuscular mycorrhizal (AM) symbiosis, formed between vascular flowering plants and fungi of the order Glomales (Smith and Read, 1997). These associations are widespread throughout the world, and both fossil evidence and molecular data indicate that the AM symbiosis existed in the earliest land plants, which underlies suggestions that the association may have enabled plants to colonize land (Pirozynski and Malloch, 1975; Remy et al., 1994; Redeker et al., 2000). The most frequently described outcomes of the AM symbiosis include a carbon supply for the fungus and an enhanced mineral nutrient supply, in particular phosphorus, for the plant. Phosphorus is an essential mineral nutrient that is frequently limiting for plant growth; consequently, the association has far-reaching effects on plant health

and subsequently on ecosystem structure (Smith and Read, 1997; van der Heijden et al., 1998).

The development of the symbiosis requires significant alterations in both symbionts that are assumed to be coordinated via reciprocal signal exchange. In general, the fungus penetrates the epidermis via an appressorium, and intercellular and intracellular growth through the outer cortex ensues. In the inner cortex, the fungus differentiates within the cortical cells, forming dichotomously branched hyphae, termed arbuscules. These elaborate structures are enveloped in an extension of the plasma membrane of the cell, and the resulting arbuscule–cortical cell interface is the site of phosphorus transfer from the fungus to the plant (Bonfante-Fasolo, 1984; Gianinazzi-Pearson, 1996). The cytoskeleton of the invaded cortical cell undergoes massive transient rearrangements, presumably to enable the development of the arbuscular interface (Genre and Bonfante, 1997, 1998, 1999; Blancaflor et al., 2001), and recent analyses suggest that reorganization may be signaled before penetration of the cell (Blancaflor et al., 2001).

Arbuscule development is accompanied by increases in the expression of genes that encode enzymes of flavonoid and jasmonic acid biosynthesis (Harrison and Dixon, 1993, 1994; Hause et al., 2002). Chitinase, glutathione *S*-transferase, proline-rich protein, sugar transporter, arabinogalactan protein, and tubulin transcripts also are increased in these cells (Bonfante et al.,

¹ To whom correspondence should be addressed. E-mail mjh78@cornell.edu; fax 607-254-6779.

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1996; Harrison, 1996; Strittmatter et al., 1996; van Buuren et al., 1999; Salzer et al., 2000; Journet et al., 2001; Wulf et al., 2003). The roles of these gene products in arbuscule development or activity are mostly unknown, but it is predicted that some of them function in the development of the symbiotic interface, either in the matrix or in the periarbuscular membrane. Phosphate transporters expressed exclusively in the symbiosis have been described in potato, rice, and *Medicago truncatula* (Rausch et al., 2001; Harrison et al., 2002; Paszkowski et al., 2002), and in the latter species, the phosphate transporter was shown to be located in the periarbuscular membrane, where it is assumed to play a role in the uptake of phosphate released from the arbuscule. Despite the continual invasion of cortical cells and extensive levels of fungal biomass that can accumulate within the root, plant defense responses generally are not induced significantly. Small, transient increases in defense gene transcripts have been observed in the initial stages of the symbiosis, but these decline as the symbiosis develops (Gianinazzi-Pearson et al., 1992; Harrison and Dixon, 1993; Lambais and Mehdy, 1993; Kapulnik et al., 1996).

Relatively little is known about the signal molecules involved in plant-AM fungal communication. Secondary metabolites, including flavonoids and isoflavonoids, have been implicated in plant fungal signaling but do not appear to be absolutely required for the development of the symbiosis (Siqueira et al., 1991; Poulin et al., 1993; Bécard et al., 1995; Phillips and Kapulnik, 1995; Xie et al., 1995; Poulin et al., 1997; Akiyama et al., 2002). A tomato mutant impaired in its ability to elicit spore germination may provide information on these early signaling events (David-Schwartz et al., 2001). There is evidence for a mobile fungal signal that elicits changes in plant gene expression before contact with the root (Kosuta et al., 2003) and also evidence that appressoria form in response to a plant signal endogenous to the epidermal cell wall (Nagahashi and Douds, 1997). However, the nature of the signal molecules involved remains unknown. Plant hormones may mediate some of the downstream developmental changes, but to date, causal relationships have not been established (van Rhijn et al., 1997; Barker and Tagu, 2000; Hause et al., 2002; Shaul-Keinan et al., 2002). Although the signals are unknown, in legumes, one component of a signaling pathway required for the development of both the mycorrhizal and rhizobium/legume symbioses has been identified. Mutants lacking the NOR/SYMRK Leu-rich repeat receptor kinase are unable to establish symbioses with either rhizobium or mycorrhizal fungi, and the fungi are restricted largely to the epidermis (Endre et al., 2002; Stracke et al., 2002). Other loci required for both symbioses have been identified genetically and may encode additional components of a nodule/mycorrhizal signaling cascade (Gianinazzi-Pearson et al., 1991; Sagan et al., 1995; Wegel et al., 1998).

To date, the molecular mechanisms that underlie the development of symbiosis remain poorly understood. Only a few of the genes that play roles in the development of the AM symbiosis have been identified. Genomics programs, particularly those focused on the model legumes *M. truncatula* and *Lotus japonicus*, have resulted in a tremendous increase in symbiosis-associated sequence information, and by electronic and molecular analyses, the cataloging of the transcriptomes of nodules and mycorrhizal roots is progressing (Fedorova et al.,

2002; Journet et al., 2002; Wulf et al., 2003). Here, we used cDNA arrays and expression profiling to obtain insights into the transcriptional alterations associated with the development of a mycorrhizal symbiosis between *M. truncatula* and an AM fungus, *Glomus versiforme*. Sixty-seven genes whose expression is altered significantly in the AM symbiosis were identified, along with an additional 25 genes that show transcriptional changes in response to alterations in phosphate nutrition. The majority of these genes were not known previously to be regulated in response to the development of an AM symbiosis, and clusters of genes with distinct expression patterns were identified. Included in this set are genes that encode components implicated in the biosynthesis of the periarbuscular matrix as well as a significant number of proteins potentially involved in transcriptional regulation and signaling. The spatial expression patterns of two genes expressed only in the AM symbiosis were analyzed in transgenic roots. These showed unique cell type-specific expression patterns that suggest activation by distinct signals and support roles in overlapping developmental phases of the symbiosis.

RESULTS

ESTs from Mycorrhizal Roots

A cDNA library was prepared from *M. truncatula* roots colonized with *G. versiforme*. Roots were sampled at 10, 17, 22, 31, and 38 days after inoculation; consequently, the library contains cDNAs representing genes expressed at all stages of development of the symbiosis and from both symbionts. A total of 7351 ESTs from this library have been deposited in GenBank and in the TIGR *M. truncatula* Gene Index. A comparison of these ESTs with the total *M. truncatula* EST collection (182,460 ESTs) revealed that the *M. truncatula/G. versiforme* library has 888 unique ESTs not found in any other libraries. The number of EST sequences from *G. versiforme* present in this library is unknown, but by extrapolating from estimates of the proportion of plant and fungal RNA in mycorrhizal RNA samples, we suggest that it is not >5%.

Arrays and Experimental Materials

To identify genes whose expression is regulated differentially in response to the development of the AM symbiosis, cDNA inserts from 2268 unique cDNA clones from the *M. truncatula/G. versiforme* library were arrayed at high density on nylon membranes. Each clone was spotted in duplicate, and the membranes each contained 768 cDNAs, 12 of which were controls. The controls included genes shown previously to be regulated in response to the development of the symbiosis. However, at the time we initiated these experiments, gene expression information was limited, and the genes reported as induced in the AM symbiosis actually showed only modest changes in transcript levels in mycorrhizal roots. Therefore, we included the AW587100 cDNA and a fragment of the AW587100 3' untranslated region (UTR) sequence as additional controls. A preliminary array experiment had indicated that AW587100 and a second gene, AW585598, showed increased transcripts in mycorrhizal

roots (J. Liu and M.J. Harrison, unpublished data). Both were present in the 2268-ungene set.

cDNA probes were prepared from replicate sets of *M. truncatula* mycorrhizal roots harvested at 8, 15, 22, 31, and 36 days after inoculation with *G. versiforme* and the corresponding mock-inoculated controls. The mycorrhizal root samples showed between 8.7 and 68.9% of the root length colonized (Table 1). In the 8-day samples, *G. versiforme* hyphae and appressoria were abundant on the surface of the root. Some of the appressoria had penetrated the root and colonization of the outer cortex had been initiated, but few or no arbuscules were present. The development of the symbiosis is not a synchronous process, and after the initial colonization of the root cortex, secondary infection events commence and the invasion process is reiterated. Consequently, the 15-, 22-, 31-, and 36-day samples contained all of the structures associated with the symbiosis as *G. versiforme* continued to spread throughout the root system. The relative amounts of RNA from *M. truncatula* and *G. versiforme* in the RNA samples prepared from these roots were estimated by RNA gel blot analysis using short species-specific internal transcribed spacer sequence probes (Maldonado-Mendoza et al., 2002). As expected, the percentage of RNA from *G. versiforme* increased with increasing colonization of the root system, reaching 4.5 and 8.1% in 36-day samples in replicate experiments 1 and 2, respectively (Table 1).

To distinguish genes that respond to colonization by *G. versiforme* from those that respond to the secondary effect of increased phosphorus nutrition, the arrays were hybridized with cDNA probes prepared from replicate sets of plants grown under low- and high-phosphate conditions. The phosphate content and transcript levels of a phosphate starvation-induced gene, *Mt4* (Burleigh and Harrison, 1997), were used as indicators of the phosphate status of the materials.

Reproducibility of Array Data

After hybridization of the arrays with probes from the replicate mycorrhiza and phosphate experiments, the signals from the duplicate spots were averaged and adjusted to account for differences in the labeling and hybridization of the probes and the amount of *M. truncatula* RNA in the mycorrhizal root samples. For each replicate experiment, the ratio of the average adjusted

signal in mycorrhizal/nonmycorrhizal roots and high/low phosphate was calculated for each gene and expressed as a log₁₀ expression ratio (LR). The reproducibility of the duplicate spots on the arrays and the variation observed between replicate experiments were assessed. The data obtained from duplicate spots on the same array were highly reproducible, and 99% of the values fell within ± 0.2 LR of the mean (Figures 1A and 1B). As expected, the data from replicate experiments showed greater variability (Figure 1C). For the three filters that encompass the 2268-gene set, a comparison of the average LRs for each spot in the two replicate experiments revealed a normal distribution, with 94% of the spots within ± 0.33 , ± 0.37 , and ± 0.34 LR of the mean for filters A, B, and C, respectively. This finding is similar to the variation noted previously on microarrays (Wang et al., 2000; Kawasaki et al., 2001). We elected to use these values as significance thresholds, and in subsequent experiments, only data for genes whose change in expression (LR) was greater than the appropriate filter threshold in both replicate experiments were considered significant.

Differential Gene Expression in the AM Symbiosis

Using the significance thresholds described above, 92 of the 2268 genes showed significant changes in expression in both replicate experiments at one or more of the time points during the development of the symbiosis or during growth under high-versus low-phosphate conditions. The expression ratios for this group of 92 genes were clustered and displayed using Cluster and Treeview software (Figure 2) (Eisen et al., 1998). Four large clusters and three smaller clusters, each with distinct expression patterns, were apparent. The expression ratios for these 92 genes and their GenBank accession numbers are available in the supplemental data online.

Cluster 1 was the largest and encompassed genes whose expression increased in mycorrhizal roots. In general, the first significant increases in transcript levels were detected at 15 or 22 days after inoculation. Transcript levels continued to increase with increasing colonization of the root system, showing maximal levels at 31 or 36 days after inoculation. The different time points are, in effect, independent measurements, and the finding that most genes showed a change over more than one adjacent time point is an additional indication of significance (Table 2). The AW587100-positive controls fell into this cluster, along with a set of 20 genes, including putative signaling components, transcription factors, proteases, structural proteins, and transporters, that were not known previously to be regulated in response to the development of the symbiosis (Table 2). AW585598, confirmed as mycorrhiza induced in preliminary experiments, actually fell just below the significance thresholds in the current array experiments. The genes in cluster 1 did not show significant increases in transcript levels during growth under high-phosphate conditions, and we conclude that their expression is regulated in response to the development of the symbiosis rather than to changes in phosphate nutrition.

To further validate these array results, six genes from this cluster were used as probes in RNA gel blot analyses. All of the genes selected showed significantly higher transcript levels in mycorrhizal roots relative to nonmycorrhizal controls, and for

Table 1. Colonization Levels and Relative Amounts of *G. versiforme* and *M. truncatula* RNA in Mycorrhizal Root Samples in Two Replicate Experiments

Days after Inoculation	Experiment 1			Experiment 2		
	RLC (%)	Gv RNA (%)	Mt RNA (%)	RLC (%)	Gv RNA (%)	Mt RNA (%)
8	8.7	2.9	97.1	11.8	1.8	98.2
15	11.2	2.3	97.7	17.4	2.5	97.5
22	39.0	5.5	94.5	43.9	3.9	96.1
31	54.6	5.1	94.9	58.9	7.1	92.9
36	60.7	4.5	95.5	68.9	8.1	91.9

Gv, *G. versiforme*; Mt, *M. truncatula*; RLC, root length colonized.

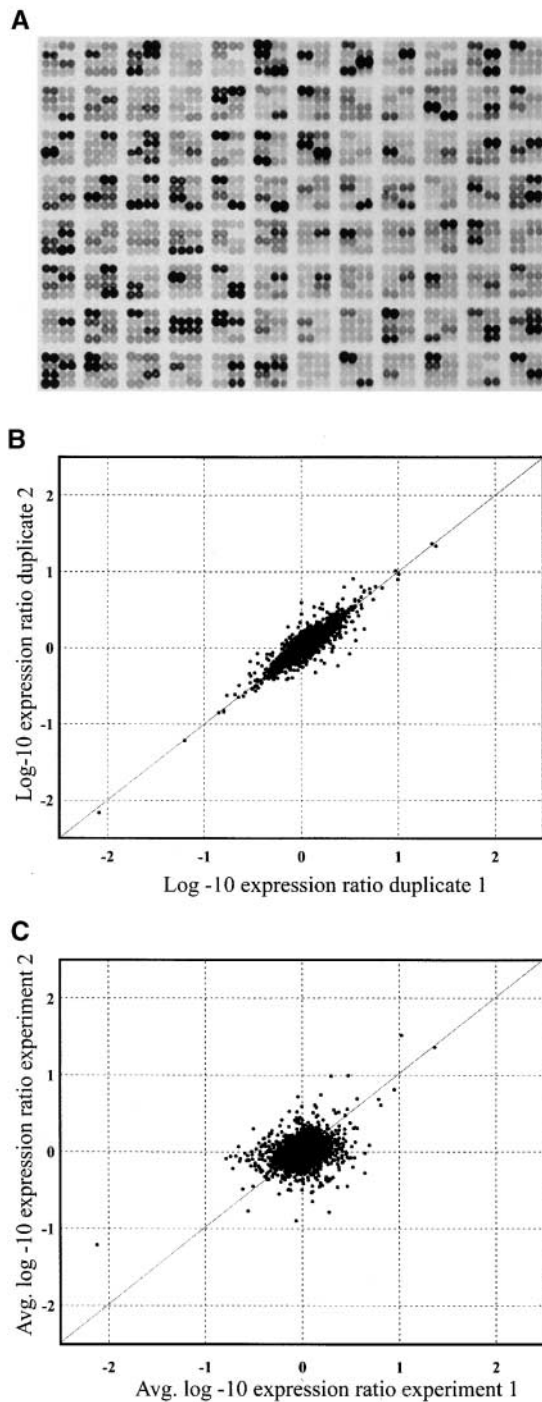


Figure 1. Array Quality and Variation within and between Experiments.

(A) TIFF image of a cDNA array hybridized with a ^{32}P -labeled cDNA probe from mock-inoculated *M. truncatula* roots harvested at 31 days after inoculation. The image shown is from filter C.

(B) Scatterplot comparing the LRs of duplicate spots on the arrays. Ninety-nine percent of the spots fall within ± 0.2 LR of the mean. The data shown are from filter C. Data from filters A and B fall within the same limits.

(C) Scatterplot comparing the average LRs from two experiments involving independent biological samples. Ninety-four percent of the

spots fall within ± 0.34 LR of the mean. The data shown are from filter C. Values from filters A and B fall within ± 0.33 and ± 0.37 LR of the mean, respectively.

five of these genes, transcripts were not detected in nonmycorrhizal roots (Figure 3A).

The origins of the genes in cluster 1 (Table 2) were evaluated. Those genes that were expressed in nonmycorrhizal roots, or that were represented by ESTs present in other nonmycorrhizal libraries in the TIGR *M. truncatula* Gene Index, can be assumed to be *M. truncatula* genes (Table 3). The 11 genes represented by singleton ESTs or mycorrhiza-specific tentative consensus could be *M. truncatula* or *G. versiforme* genes (Table 3). Evaluation of the genome of origin by genomic PCR revealed clearly that all of the ESTs except AW586753 represent *M. truncatula* genes (see supplemental data online). AW586753 shares significant identity with sequences from mycorrhizal fungi, and the corresponding gene could be amplified from genomic DNA of *G. versiforme*. The fungal origin of AW586753 and the plant origins of MtSCP1 and MtCel1 also were confirmed by sequence analysis.

In contrast to cluster 1, cluster 2 contained genes whose transcript levels were significantly higher in mycorrhizal roots at 8 days after inoculation and then declined as the symbiosis developed (Figure 2, Table 4). This group includes a number of genes predicted to encode defense- or stress-related proteins as well as genes potentially involved in stress-related signaling. Earlier studies had shown that the development of the AM symbiosis is associated with a small transient increase of defense gene expression. However, except for chalcone synthase, the genes in this cluster had not been reported previously to be expressed differentially in the AM symbiosis. RNA gel blot analyses with AW584703 and AW584415 confirmed the array results and also provided further insight into the expression patterns (Figure 3B). At 8 days after inoculation, these two genes were expressed at relatively low levels in nonmycorrhizal roots, and expression was increased in mycorrhizal roots. During the subsequent 36 days, transcript levels gradually increased in nonmycorrhizal roots but decreased in mycorrhizal roots, which explains the strongly negative LR values seen on the arrays. In general, the expression of genes in cluster 2 did not change in response to growth with high phosphate. Cluster 2 is flanked by two small clusters (clusters 3 and 4) of genes whose transcript levels also decreased in the AM symbiosis, but without significant increases at 8 days after inoculation (Table 5). Included in these clusters are genes that encode defense-related proteins, PR3 and chitinase, and three other genes that are similar to genes regulated in response to various biotic and abiotic stresses, including a stress-induced protein kinase. Again, the majority of these proteins were not known to be regulated in mycorrhizal roots, but they are consistent with the theme that defense- and stress-response transcripts decrease in mycorrhizal roots.

Genes in a third, smaller cluster, cluster 5, showed increased expression in response to the development of the symbiosis and also in response to growth in high-phosphate conditions (Table 6). Significant changes in gene expression in mycorrhizal

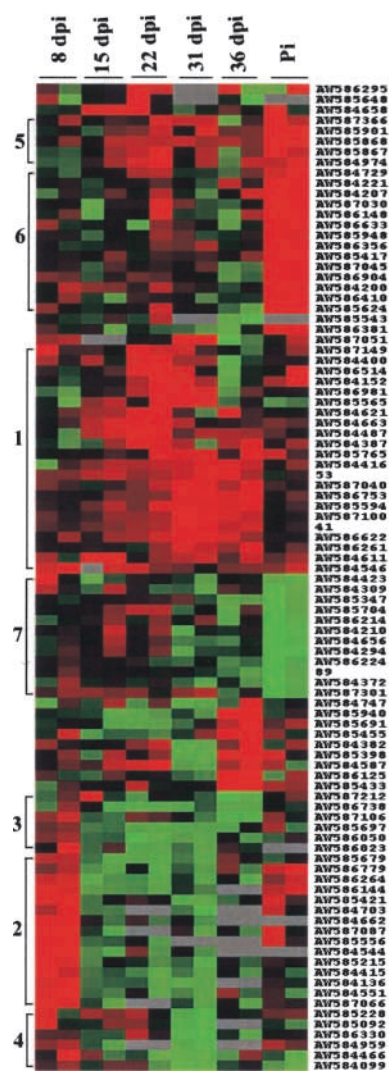


Figure 2. Cluster Analysis of 92 Transcripts during the Development of the AM Symbiosis and during Growth with 2 mM Versus 0.02 mM Phosphate.

Clustered expression ratios visualized by Treeview (Eisen et al., 1998). The rows represent the individual clones, and the GenBank numbers of the ESTs are indicated. The rows labeled 53, 41, and 89 represent the AW587100, AW587100 3' UTR, and Mt4 controls, respectively. The ratios of expression in mycorrhizal/nonmycorrhizal roots at 8, 15, 22, 31, and 36 days postinoculation (dpi) and in high/low phosphate (Pi) are represented. Biological replicates are adjacent to each other. The color reflects the LR. Red represents a positive LR, green represents a negative LR, and black represents a LR of zero. In each case, the saturation reflects the magnitude of the change. Seven groups of transcripts with distinct expression patterns are indicated.

roots were apparent at 22 days after inoculation. Arbuscules are transient structures that develop, reach maturity, and then senesce over a period of 7 to 16 days, depending on the species (Alexander et al., 1988, 1989). Given that the first arbuscules were initiated at 8 days after inoculation, the period from 15 to 22 days would coincide with the maturity of the initial

waves of arbuscules and the onset of symbiotic phosphate transport. Because these genes also were induced in response to high phosphate, it seems likely that the increase in transcript levels observed in the mycorrhizal roots was triggered as a consequence of alterations in phosphate nutrition.

Interestingly, two of the genes in this cluster (AW587366 and AW585867) share identity with genes regulated in response to senescence. The mechanisms that underlie arbuscule senescence and decay are unknown, but these genes might provide leads to an understanding of this process.

Genes Regulated in Response to Phosphate

The final two clusters contain transcripts that change significantly in response to growth under high- versus low-phosphate conditions. Genes in cluster 6 showed significant increases in transcript levels during growth in high-phosphate conditions (Table 7). Apart from a putative phosphoenolpyruvate carboxykinase, the 14 genes in this cluster were not known previously to be responsive to phosphate. Included in this cluster are two ESTs that are similar to kinases that interact with calcineurin B-like calcium sensor proteins and that have been implicated in signaling pathways involved in responses to stress, hormones, and environmental cues (Albrecht et al., 2001). A *M. truncatula* zinc transporter also is present in this cluster and shows a 10-fold increase in transcripts. Transcriptional profiling has revealed overlaps in responses to mineral nutrient stresses (Wang et al., 2001, 2002), and in barley, zinc nutrition is known to affect the expression of phosphate transporter genes (Huang et al., 2000). Our data suggest that the opposite effect also occurs and that changes in phosphorus nutrition affect the expression of a zinc transporter.

RNA gel blot analysis with one representative of this cluster, AW587045, confirmed that transcript levels for this gene increase in response to phosphate (Figure 3C). In general, genes in this group showed no significant changes in response to the development of the AM symbiosis, although there are some exceptions. AW586356 showed a marginally significant change in transcript level at 22 days after inoculation in mycorrhizal roots. The LR for one of the replicates was below the assigned significance threshold. However, RNA gel blot analyses (Figures 3A and 3C) confirmed the phosphate-induced expression patterns and also demonstrated mycorrhiza-induced expression.

By contrast with cluster 6, genes represented in cluster 7 showed a decrease in transcript levels under high-phosphate conditions, and the expression of some of these genes was downregulated in the AM symbiosis (Table 8). A well-described phosphate starvation-induced gene, *Mt4*, falls into this cluster, as does a putative acid phosphatase (Baldwin et al., 2001). As seen with the genes in cluster 7, some members of this group showed marginally significant changes in mycorrhizal roots that require additional validation to be conclusive. Small, localized decreases in transcripts in mycorrhizal roots may be difficult to detect with the arrays.

Specificity of Expression of Mycorrhiza-Induced Genes

Legumes have the unique ability to form symbioses with nitrogen-fixing bacteria as well as mycorrhizal fungi, and there is evi-

Table 2. Transcripts Upregulated in Mycorrhizal Roots (Cluster 1)

Accession No.	Current Annotation	LR ^a					
		8	15	22	31	36	Pi
AW584621	No hit	-0.05/0.14	-0.05/0.26	0.47/0.57	0.11/-0.23	0.60/0.10	0.01/-0.04
AW584663	Unknown protein	-0.01/0.07	0.21/0.28	0.46/0.34	0.22/0.01	0.05/0.07	0.11/0.09
AW584487	TINY-like protein	-0.01/-0.18	0.22/0.29	0.35/0.42	0.10/0.21	0.19/0.17	0.09/0.01
AW584387	Mitotic checkpoint protein	-0.06/-0.23	-0.13/0.21	0.52/0.36	0.24/-0.2	0.25/0.38	0.05/0.21
AW585765	Cys proteinase	-0.08/0.05	-0.03/0.22	0.08/1.0	0.79/0.82	0.16/0.99	0.71/0.21
AW584416	Unknown protein	-0.43/0.15	0.24/0.40	0.37/0.48	0.49/0.61	0.60/0.50	0.03/0.27
AW587040	Ripening-related protein	0.29/0.20	-0.14/0.16	0.30/0.59	0.97/0.78	0.47/0.99	0.19/0.07
AW586753	Surface antigen p2 protein	0.15/0.10	-0.01/0.42	0.42/0.46	1.18/1.31	1.03/1.52	-0.01/0.37
AW585594	Transfactor-like protein	0.21/0.14	0.35/0.74	0.40/1.18	1.10/1.05	0.99/1.14	-0.08/0.27
AW587100	MtSCP1	0.31/0.12	0.20/0.46	0.45/0.53	1.06/1.14	0.79/0.69	-0.07/0.10
AW586622	Ser carboxypeptidase	0.04/0.33	0.10/0.33	0.37/0.53	0.85/0.64	0.57/0.31	0.06/0.01
AW586261	Ripening-related protein	0.08/0.08	0.14/0.11	-0.19/0.44	0.83/0.70	0.45/1.65	-0.01/0.31
AW584611	Subtilisin-like protease	0.26/-0.11	0.54/0.50	0.05/0.23	0.31/0.39	0.14/0.36	0.12/0.25
AW587149	Trichohyalin-like protein	0.50/0.11	0.11/-0.02	0.66/0.39	0.39/0.11	-0.04/-0.20	0.05/0.23
AW584400	Unknown protein	0.29/0.07	0.31/-0.07	0.47/0.59	0.27/0.26	-0.64/-0.12	0.07/0.04
AW586514	MTD1 protein	0.07/-0.08	0.02/0.10	0.36/0.37	0.31/0.02	-0.29/-0.05	-0.03/0.37
AW586981	DnaJ-like protein	-0.22/-0.36	0.03/0.12	0.40/0.79	0.64/0.54	-0.35/0.20	0.03/0.03
AW585565	Glucosyltransferase 3	-0.04/-0.36	0.18/0.27	0.003/0.4	0.43/0.40	-0.16/0.05	-0.23/-0.18
AW584152	CCCH-type zinc finger	0.04/-0.17	0.06/0.04	0.35/0.69	0.54/0.31	-0.39/-0.08	0.32/0.61
AW584546	Sugar transporter	0.42/0.72	F ^b /0.44	0.43/0.22	0.16/-0.01	0.19/0.20	0.21/0.41
AW587100	Control MtSCP1	0.34/0.37	0.64/0.75	0.50/0.58	0.75/1.02	1.09/0.56	-0.17/0.20
AW587100	3' UTR of MtSCP1	0.24/0.18	0.25/0.34	0.45/0.51	0.87/1.14	0.56/0.69	-0.05/0.14

^a LR values are for experiment 1/experiment 2 at 8, 15, 22, 31, and 36 days after inoculation and in high/low phosphate (Pi). LR exceeding significance thresholds (0.33, 0.37, or 0.34 LR for filters A, B, and C, respectively) are shown in boldface.

^b F, EST for which LR values in replicate experiments were highly variable (>0.6 LR).

dence for components of signaling pathways and symbiotic interfaces common to both symbioses (Perotto et al., 1994; Journet et al., 2001; Endre et al., 2002; Stracke et al., 2002). RNA gel blot and reverse transcriptase-mediated (RT) PCR analyses were used to assess the expression of five of the mycorrhiza-induced genes in nodulated roots. Leghemoglobin was included as a control. As expected, leghemoglobin transcripts were abundant in nodulated roots. Transcripts corresponding to a putative Cys proteinase (AW585765) also were present in nodulated roots, whereas the remaining four mycorrhiza-induced genes were not expressed in nodulated roots (Figures 4A and 4B).

The expression profiles obtained from the arrays and the RNA gel blot analyses also were consistent with the EST distribution and frequency within libraries in the TIGR *M. truncatula* Gene Index. The index contains 182,460 *M. truncatula* ESTs distributed among 38 libraries. The Cys proteinase is present exclusively in the mycorrhizal root and nodule libraries, and the remaining four genes are represented only in the mycorrhizal root libraries. Overall, computer analyses suggest that 50% of the genes from cluster 1 are represented only in the libraries prepared from mycorrhizal roots (Table 3).

Cell Type-Specific Expression Patterns of Two Mycorrhiza-Induced Genes, *MtSCP1* and *MtCel1*

The arrays enabled the identification of novel genes whose expression is regulated during the development of the symbiosis. However, because the colonization process is asynchronous,

information concerning the spatial expression patterns cannot be obtained from these analyses. With the goal of understanding the role of these genes in the development of the symbiosis, we used promoter-reporter gene fusions to gain insight into the spatial expression patterns of two novel mycorrhiza-induced genes, *MtSCP1* (AW587100) and *MtCel1* (AW585598).

The cDNA clone from which the *MtSCP1* EST sequence (AW587100) was derived was a full-length clone and was designated *MtSCP1*. The cDNA is predicted to encode a protein of 495 amino acids that shares 53 to 56% identity with Ser carboxypeptidase II proteins from barley, wheat, and Arabidopsis (Breddam and Ottesen, 1987; Soerensen et al., 1987; Degan et al., 1994; Li et al., 2001). The AW585598 EST was derived from a partial cDNA of 1408 bp. A combination of library screening and RT-PCR analysis enabled the identification of a genomic clone and subsequently a full-length cDNA clone designated *MtCel1*. The *MtCel1* cDNA is predicted to encode a protein of 601 amino acids that shares 43 to 45% identity with membrane-anchored endo-1,4-β-D-glucanases (EGases; EC 3.2.1.4), Arabidopsis KOR1, *Brassica napus* Cel16, barley Cel1, and tomato Cel3.

To examine the expression patterns of the *MtSCP1* and *MtCel1* genes, DNA regions 5' of the respective open reading frames were fused to the *uidA* and green fluorescent protein (GFP) reporter genes, and *M. truncatula* plants containing transgenic roots carrying these constructs were created (Boisson-Dernier et al., 2001). The plants were inoculated with *G. versiforme*, and after the development of the symbiosis, the roots were examined for the expression of the reporter genes.

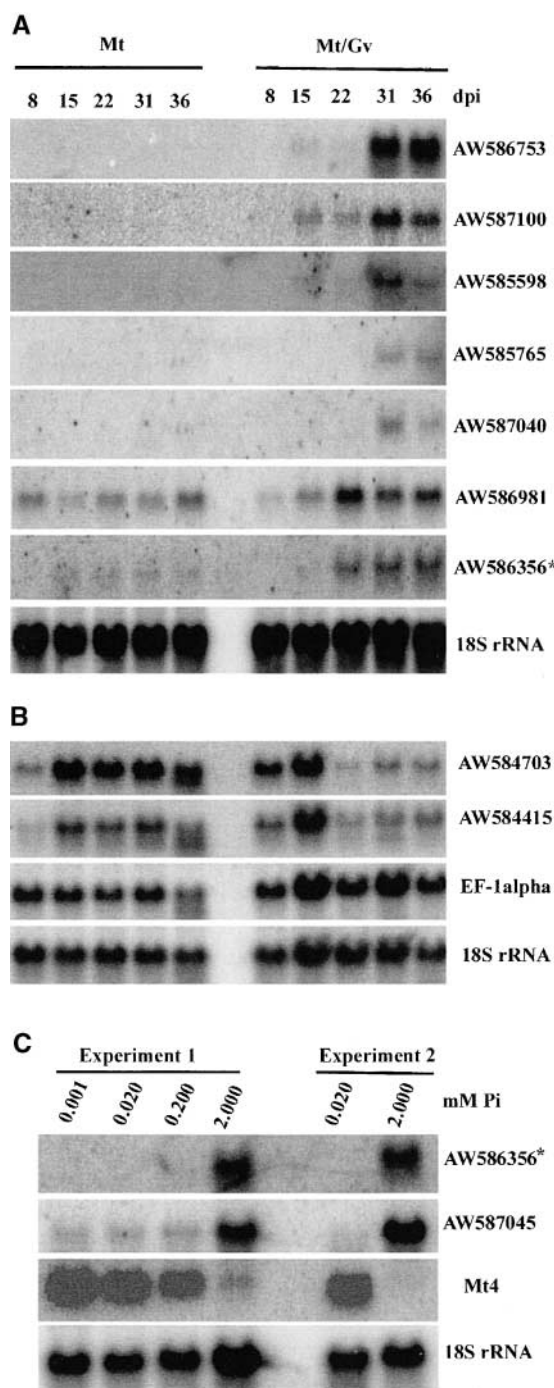


Figure 3. RNA Gel Blot Analysis of a Selection of the Genes Identified on the Arrays.

(A) and **(B)** RNA gel blot analysis with total RNA (12 µg) from noncolonized *M. truncatula* roots (Mt) and *M. truncatula* roots colonized with *G. versiforme* (Mt/Gv) at 8, 15, 22, 31, and 36 days postinoculation (dpi). The blots were probed, stripped, and reprobated sequentially with the probes indicated at right. All of the genes except AW586356 are members of cluster 1. AW586356 is a member of cluster 5.

(C) RNA gel blot analysis with root RNA (12 µg) from *M. truncatula* plants grown for 31 days (experiment 1) and 42 days (experiment 2) with

In the transgenic roots carrying the *MtCel1* promoter–GFP fusion, strong green fluorescence was visible exclusively in cells containing arbuscules (Figures 5A and 5B). This pattern of expression was confirmed in transgenic roots carrying the *MtCel1* promoter–β-glucuronidase (GUS) fusion, in which strong blue staining, indicating the presence of GUS activity, was visible in the cortex (Figure 5C) and was associated specifically with cells containing arbuscules (Figures 5D and 5E). Reporter gene expression was not visible in epidermal cells or in cells through which an intracellular hypha had passed, suggesting that expression is correlated tightly with arbuscule development. This pattern is the same as that reported recently for a *M. truncatula* mycorrhiza-specific phosphate transporter (Harrison et al., 2002) and may reflect the presence of a cell-autonomous signal that induces the expression of genes associated with arbuscule development and function.

By contrast, transgenic roots expressing the *MtSCP1* promoter–GFP fusion showed strong green fluorescence in the cortical cells containing arbuscules and also in adjacent cells in the cortex (Figures 6A and 6B). In many instances, GFP was visible in four or five cells in a cell file, in which only one or two cells in the file contained an arbuscule. This expression pattern is consistent with a response to a mobile, cell-nonautonomous signal (Figures 6A to 6D). Occasionally, weak GFP was visible in outer cortical cells, but only in regions of the root in which the inner cortex was colonized (Figures 6C and 6D). Confocal microscopy analyses revealed the presence of GFP in the outer cortical cells that had been penetrated by a fungal hypha, and expression coincided with the path taken by the fungus to the inner cortex (Figures 6E and 6F). We conclude that expression in the outer cortical cells was transient, because as arbuscules developed in the inner cortical cell layers, green fluorescence was very weak or no longer visible in the outer cortical cell layers, although the fungal hyphae still were present in these cells (Figures 6C and 6D). These expression patterns were confirmed in transgenic roots carrying the *MtSCP1* promoter–GUS fusion (data not shown). In both sets of transgenic roots, *MtSCP1* expression was not observed in any of the epidermal cells lying under the appressoria, although epidermal cells that had been penetrated by fungal hyphae were present in these samples. The expression patterns of these two genes are consistent with the presence of both cell-autonomous and cell-nonautonomous signals operating in mycorrhizal roots.

DISCUSSION

cDNA arrays were used to monitor transcript levels in mycorrhizal roots and as a gene discovery tool to identify genes whose expression is regulated during the development of an AM symbiosis. From a set of 2268 cDNAs, we identified 67

differing levels of phosphate fertilization. The blots were probed, stripped, and reprobated sequentially with the probes indicated at right. The *Mt4* gene was included as a control, and transcript levels reflect the degree of phosphate starvation (Burleigh and Harrison, 1998).

Table 3. Frequency and Distribution of ESTs Representing Mycorrhiza-Induced Genes in the *M. truncatula* Gene Index (TIGR)

Accession No.	Current Annotation	TC No. ^a	Myc 1	Myc 2	Nod	Nem	Path	Others
AW585594	Transfactor-like protein	45467	4	6	0	0	0	0
AW587100	MtSCP1	53843	4	26	0	0	0	0
AW586622	Ser carboxylase II-3 protein	53844	2	4	0	0	0	0
AW585598	MtCel1	63534	1	1	0	0	0	0
AW586261	Putative ripening-related protein	66692	1	1	0	0	0	0
AW587040	Putative ripening-related protein	Singleton	1	0	0	0	0	0
AW586753	Surface antigen p2 protein	Singleton	1	0	0	0	0	0
AW584387	Mitotic checkpoint protein	Singleton	1	0	0	0	0	0
AW584611	Subtilisin-like protease	Singleton	1	0	0	0	0	0
AW587149	Trichohyalin-like protein	Singleton	1	0	0	0	0	0
AW584658	Putative disease resistance gene	Singleton	1	0	0	0	0	0
AW585765	Cys proteinase	68397	2	2	14	0	0	0
AW584416	Unknown protein	68368	1	7	2	0	0	1
AW584663	Unknown protein	71847	1	0	2	0	0	1
AW585565	Glucosyltransferase-3 protein	70387	2	0	3	0	0	4
AW584152	CCCH-type zinc finger protein	60585	4	0	3	1	1	6
AW584487	TINY-like protein	69555	1	3	1	0	3	4
AW584400	Unknown protein	61629	2	0	2	0	0	5
AW584621	No hit	71807	1	0	0	0	1	3
AW586514	MTD1 protein	68764	3	1	3	0	2	11
AW586981	DnaJ-like protein	60009	3	2	0	1	2	16
AW584546	Sugar transporter	68381	2	0	6	2	5	29

Myc 1, cDNA libraries *M. truncatula*/G. *versiforme* (MHAM) and *M. truncatula*/G. *intraradices* (MtBC); Myc 2, suppressive subtractive hybridization cDNA libraries *M. truncatula*/G. *intraradices* (MTGIM and MTAMP) and *M. truncatula*/G. *mosseae*; Nod, cDNA libraries *M. truncatula*/S. *meliloti* (KV1, KV2, KV3, GVN, GVSJ, and MtBB); Nem, cDNA library *M. truncatula*/Meloidogyne *incognita* (BNIR); Path, cDNA libraries *M. truncatula*/Colletotrichum *trifolii* (DSIL) and *M. truncatula*/Phytophthora *medicaginis* (DSIR); Others, all other *M. truncatula* cDNA libraries in the *M. truncatula* Gene Index (TIGR) except the 14 libraries described above.

^aTC, TIGR tentative consensus sequence.

genes that were regulated differentially during the development of the symbiosis. Of these genes, 20 showed substantial increases in transcript levels that correlated with the proliferation of the fungus within the roots. Ninety-five percent of the mycorrhiza-regulated genes were not known previously to be reg-

ulated in the AM symbiosis and provide insights into the complex array of transcriptional changes that underlie the development of the AM symbiosis.

The criterion selected for ascribing significance to changes in gene expression on the arrays was relatively stringent, and it is

Table 4. Transcripts Upregulated Transiently in the Initial Stages of the Mycorrhizal Symbiosis (Cluster 2)

Accession No.	Current Annotation	LR ^a					
		8	15	22	31	36	Pi
AW585679	Zinc finger ankyrin	0.45/0.47	-0.28/-0.56	-0.61/-0.81	-0.32/-0.47	0.25/0.06	-0.22/0.05
AW586264	Acid phosphatase	0.41/0.45	-0.42/-0.30	-0.30/-0.37	-0.10/-0.36	0.02/-0.42	0.21/0.40
AW586144	Ubiquitin-like protein	0.42/0.37	-0.28/-0.26	-0.24/-0.44	-0.10/-0.22	F ^b /F	0.28/0.37
AW585421	CHS protein	0.35/0.45	-0.16/-0.003	0.08/0.01	-0.27/-0.33	-0.14/-0.01	0.30/0.09
AW584703	Phi-1 protein	0.36/0.81	-0.28/-0.31	F/F	-0.27/-0.84	F/F	0.46/-0.04
AW584662	HMGR-CoA reductase	0.60/0.68	0.06/-0.19	0.11/-0.20	0.01/-0.25	F/F	F/F
AW587087	Unknown protein	0.40/0.36	-0.07/-0.25	F/F	0.07/-0.35	0.01/-0.18	0.31/-0.03
AW585556	SRC2 protein	0.74/0.55	-0.19/-0.15	0.03/-0.31	F/F	F/F	0.42/0.10
AW584544	HMGR-CoA reductase	0.72/0.50	-0.25/-0.34	0.20/-0.28	0.005/-0.26	F/F	F/F
AW585215	Unknown protein	0.55/0.36	-0.21/0.02	-0.06/-0.22	-0.14/-0.50	0.04/-0.14	0.08/-0.001
AW584415	MAP kinase	0.69/0.44	-0.24/-0.17	-0.28/-0.45	-0.25/-0.61	0.004/-0.32	0.07/-0.22
AW584136	MAP kinase	0.37/0.48	-0.18/-0.15	-0.14/-0.42	-0.35/-0.48	F/F	0.10/-0.07
AW584551	Unknown protein	0.48/0.59	-0.18/-0.26	0.06/-0.36	-0.26/-0.53	0.24/-0.18	0.12/-0.28
AW587066	AGP protein	0.49/0.47	-0.12/-0.47	F/F	-0.12/-0.54	-0.004/-0.49	0.12/0.07

^aLR values are for experiment 1/experiment 2 at 8, 15, 22, 31, and 36 days after inoculation and in high/low phosphate (Pi). LR exceeding significance thresholds (0.33, 0.37, or 0.34 LR for filters A, B, and C, respectively) are shown in boldface.

^bF, EST for which LR values in replicate experiments were highly variable (>0.6 LR).

Table 5. Transcripts Downregulated in Mycorrhizal Roots (Clusters 3 and 4)

Accession No.	Current Annotation	LR ^a					
		8	15	22	31	36	Pi
AW585697	PVPR3 protein	0.14/0.20	-0.21/-0.19	-0.47/-0.53	-0.31/-0.29	-0.31/0.10	0.25/-0.20
AW586050	Unknown protein	0.21/0.31	-0.21/-0.18	-0.75/-0.44	-0.34/-0.51	0.07/-0.33	-0.19/0.10
AW586023	Protein kinase	-0.01/0.43	-0.15/0.11	-0.44/-0.41	-0.16/-0.40	0.08/-0.07	F ^b /F
AW587212	Unknown protein	-0.15/0.11	0.35/-0.08	-0.22/0.01	-0.04/-0.12	-0.51/-0.45	-0.02/0.14
AW586738	Phosphoenolpyruvate carboxylase	-0.19/0.19	0.02/-0.28	-0.31/-0.32	-0.35/-0.12	-0.37/-0.36	-0.04/-0.09
AW587106	Stress-induced kinase	0.13/0.23	-0.09/-0.12	0.09/0.08	0.03/-0.13	-0.35/-0.36	0.12/-0.03
AW585228	T13D8.8 protein	0.34/-0.12	0.21/0.15	0.23/-0.02	-0.46/-0.52	-0.01/-0.05	0.12/0.14
AW585092	Chitinase	0.38/-0.05	-0.05/0.16	0.15/-0.04	-0.43/-0.45	F/F	-0.06/-0.18
AW586330	Unknown protein	0.09/0.10	-0.11/-0.11	0.25/0.05	-0.44/-0.51	-0.06/-0.24	-0.03/0.03
AW584959	No hit	0.18/0.25	-0.12/-0.39	F/F	-0.45/-0.50	F/F	0.24/0.12
AW584099	Bifunctional nuclease	0.10/0.23	-0.09/0.14	0.14/-0.25	-0.58/-0.55	-0.12/0.003	-0.26/-0.42
AW584466	2,4-D-inducible GST	0.07/0.58	0.18/-0.21	-0.12/-0.36	-0.35/-0.38	-0.27/0.20	-0.13/-0.36

^a LR values are for experiment 1/experiment 2 at 8, 15, 22, 31, and 36 days after inoculation and in high/low phosphate (Pi). LR exceeding significance thresholds (0.33, 0.37, or 0.34 LR for filters A, B, and C, respectively) are shown in boldface.

^b F, EST for which LR values in replicate experiments were highly variable (>0.6 LR).

likely that some differentially regulated genes have been overlooked. However, the application of these significance thresholds resulted in a robust data set, and genes that were tested subsequently showed the expression patterns predicted from the arrays. In addition, the arrays contained a number of unanticipated internal controls that further validated the results. Some of the ESTs described initially in the TIGR *M. truncatula* Gene Index as singletons were shown later to represent the same gene. For example, AW584415 and AW584136 represent a single mitogen-activated protein kinase gene. They are both present on the arrays and cluster together in cluster 2. Likewise, AW584210 and AW584656 represent a putative acid phosphatase gene, and both cluster together in cluster 7.

The development of the AM symbiosis is not a synchronous process, so temporal differences in expression observed in the 15- to 36-day samples should be interpreted cautiously. In spite of this caveat, clusters of genes with distinct expression patterns were apparent. One theme that emerged from these analyses relates to defense-response genes. A significant number of genes that share sequence similarity with defense- or stress-regulated genes, including a chitinase, a pathogenesis-related (PR) protein, a putative defense-associated acid phosphatase, chalcone synthase, a stress-related mitogen-activated

protein kinase, an SRC2 homolog, two 3-hydroxy 3-methyl glutaryl-CoA reductases, and a defense-related AGP, were found in cluster 2. Additional defense- and stress-related genes were present in clusters with related expression patterns, clusters 3 and 4. Previous analyses had shown that defense-response genes, largely those that encode PR proteins and enzymes of phytoalexin biosynthesis, show transient increases in expression during the early stages of the symbiosis and then the transcript levels subsequently decline (Spanu et al., 1989; Harrison and Dixon, 1993, 1994; Gianinazzi-Pearson et al., 1996; Kapulnik et al., 1996).

The expression patterns observed here are in accord with the earlier analyses and extend them to include a much broader array of defense- and stress-response genes. Furthermore, these data indicate that some defense-response genes do not show an initial transient increase in expression but simply are downregulated as the symbiosis develops, suggesting that at least two different signals regulate the expression of defense-response genes in the AM symbiosis. In addition to genes that encode defense-response proteins, there are a number of genes that encode putative defense-associated signal transduction components in cluster 2, including a protein phosphatase, a mitogen-activated protein kinase, a Leu-rich repeat kinase, and a

Table 6. Transcripts Upregulated in Both Mycorrhizal Roots and Roots Grown in High Phosphate (Cluster 5)

Accession No.	Current Annotation	LR ^a					
		8	15	22	31	36	Pi
AW587366	Copper chaperone homolog	0.03/0.27	-0.02/0.11	0.1/0.42	0.36/0.36	-0.45/0.12	0.55/0.32
AW585901	Acyl-CoA oxidase	-0.06/0.03	-0.14/0.14	0.16/0.32	0.04/0.36	0.13/0.16	0.55/0.45
AW585868	Unknown protein	-0.01/0.23	0.01/0.36	0.38/0.35	0.43/0.28	0.33/0.21	0.55/0.50
AW585867	Ntdin protein	-0.14/-0.18	-0.03/0.27	0.39/0.59	0.05/0.33	-0.30/0.20	0.69/0.39
AW584974	Wound-induced protein	-0.13/-0.14	0.07/0.22	0.35/0.47	-0.03/0.25	-0.19/0.31	0.28/0.35

^a LR values are for experiment 1/experiment 2 at 8, 15, 22, 31, and 36 days after inoculation and in high/low phosphate (Pi). LR exceeding significance thresholds (0.33, 0.37, or 0.34 LR for filters A, B, and C, respectively) are shown in boldface.

Table 7. Transcripts Upregulated in Roots Grown in High Phosphate (Cluster 6)

Accession No.	Current Annotation	LR ^a					Pi
		8	15	22	31	36	
AW584729	Unknown protein	-0.08/-0.19	-0.01/0.03	0.31/ 0.35	-0.10/-0.11	-0.19/0.18	0.42/0.88
AW584221	Ubiquitin carrier	-0.04/0.06	-0.10/0.004	0.04/0.10	-0.02/-0.12	0.28/0.07	0.43/0.64
AW584207	Phosphoenolpyruvate carboxykinase	-0.17/0.07	-0.08/0.12	-0.04/0.24	-0.05/-0.20	0.002/0.32	0.44/0.50
AW587030	Unknown protein	-0.02/0.03	-0.35/0.05	0.06/ 0.41	0.14/-0.11	-0.15/0.004	0.46/0.55
AW586148	CIPK25	-0.05/0.12	-0.26/0.02	-0.01/0.34	-0.08/-0.10	-0.25/0.23	0.46/0.38
AW586633	Hypothetical protein	0.18/-0.07	-0.04/0.02	-0.23/0.14	0.08/0.02	-0.21/-0.05	0.49/0.37
AW585948	Putative protein	0.06/0.06	-0.15/-0.01	-0.04/0.22	0.13/-0.11	-0.08/0.07	0.78/0.38
AW585417	Zinc transporter protein	0.05/0.08	-0.02/0.22	-0.08/0.13	0.11/0.14	0.05/0.25	0.99/0.74
AW587045	Unknown protein	0.07/0.12	-0.21/0.17	0.05/0.10	0.01/0.18	-0.35/-0.26	0.95/0.81
AW586904	CIPK25	0.13/0.10	-0.10/0.05	0.12/0.09	-0.01/-0.03	-0.19/-0.12	0.44/0.47
AW584200	Fe-superoxide dismutase precursor	-0.04/0.25	0.17/0.15	0.07/0.25	0.08/0.06	-0.10/0.02	0.59/0.36
AW586410	Putative invertase	-0.02/0.12	-0.06/-0.16	0.05/0.04	0.01/-0.13	-0.05/-0.05	0.38/0.34
AW585624	Origin recognition complex subunit	0.21/-0.01	-0.08/-0.03	0.04/0.36	0.01/0.04	-0.18/ -0.48	0.62/0.50
AW586381	Stress-regulated gene	0.11/-0.05	0.05/ -0.37	-0.05/-0.1	-0.14/-0.22	-0.28/-0.04	0.37/0.51
AW586356	No hit	0.05/-0.21	-0.17/0.08	-0.11/0.31	0.28/ 0.54	-0.13/0.10	1.36/1.36

^aLR values are for experiment 1/experiment 2 at 8, 15, 22, 31, and 36 days after inoculation and in high/low phosphate (Pi). LR exceeding significance thresholds (0.33, 0.37, or 0.34 LR for filters A, B, and C, respectively) are shown in boldface.

putative RING zinc finger ankyrin protein that shares identity with a protein involved in the ubiquitination of the Ser/Thr receptor-like kinase, Xa-21. The distributions of ESTs for these genes indicate that they are expressed broadly in *M. truncatula* tissues; therefore, they are not representatives of mycorrhiza-specific signaling pathways. However, they may provide clues to the mechanisms by which defense responses are downregulated in the AM symbiosis.

Among the genes induced specifically in the AM symbiosis but not in response to phosphate (cluster 1) are two genes (AW586261 and AW587040) that encode unknown proteins that are similar to genes induced in grapes during fruit ripening (Davies and Robinson, 2000). At first glance, these events appear very different; however, some of the underlying physiological changes may be similar. Both processes are accompanied

by significant modifications to cell wall architecture (Bonfante-Fasolo et al., 1981; Bonfante and Perotto, 1995; Davies and Robinson, 2000). The functions of the grape and *M. truncatula* proteins are unknown, but they are predicted to contain cleavable signal peptides and are potentially located in the cell wall.

In addition to these proteins, a third gene, *MtCel1*, is predicted to be involved in cell wall modifications and is induced specifically in mycorrhizal roots. The *MtCel1* gene, *MtCel1*, product shares identity with members of the E-type EGase subfamily III (Brummell et al., 1997), including the Arabidopsis KOR1 protein (Nicol et al., 1998; Zuo et al., 2000), tomato Cel3 (Brummell et al., 1997), *B. napus* Cel16, and barley Cel1. These EGases are distinct in that they lack the typical eukaryotic cleavable signal peptide found in other EGases and are predicted to be type-II integral membrane proteins. Tomato Cel3, the first member of

Table 8. Transcripts Downregulated in Roots Grown in High Phosphate (Cluster 7)

Accession No.	Current Annotation	LR ^a					Pi
		8	15	22	31	36	
AW584423	Shaggy-like kinase	0.49/-0.02	-0.33/-0.12	0.06/0.02	-0.08/-0.28	-0.03/-0.05	-0.46/-0.53
AW584309	Al-induced gene	0.27/0.06	-0.16/0.28	0.05/-0.16	-0.14/-0.30	0.11/0.06	-0.62/-0.45
AW585347	Thaumatin-like gene	-0.01/-0.12	-0.02/0.02	0.08/0.09	0.16/-0.14	-0.52/-0.32	-0.77/-0.50
AW585704	α-Fucosidase	-0.02/0.02	0.03/0.20	0.37/0.36	-0.19/-0.03	-0.44/0.13	-0.71/-0.53
AW586214	Unknown protein	-0.17/-0.05	0.07/0.22	-0.08/0.21	-0.05/0.20	-0.37/0.05	-0.81/-0.62
AW584210	Acid phosphatase	0.03/0.08	-0.03/ 0.40	0.05/0.24	-0.44/-0.06	-0.09/0.07	-0.71/-0.68
AW584656	Acid phosphatase	-0.14/0.12	-0.04/0.37	0.05/0.17	-0.30/-0.26	-0.33/-0.08	-1.00/-0.79
AW584294	Ascorbate peroxidase	-0.12/0.14	0.02/0.22	0.04/0.19	-0.38/-0.14	-0.13/-0.06	-0.81/-0.71
AW584372	Phospholipase D	-0.13/0.21	-0.06/0.04	-0.07/0.06	-0.14/-0.27	-0.06/-0.23	-0.59/-0.46
AW587301	Superoxide dismutase	0.09/0.20	0.21/0.15	0.19/-0.05	0.18/0.28	-0.18/0.05	-0.62/-0.48
AW586224	Control Mt4 gene	-0.02/ 0.36	0.09/-0.03	0.002/-0.14	-0.02/-0.21	-0.56/-0.78	-2.12/-1.21
AW586224	Mt4 gene	0.08/0.18	0.08/0.03	0.14/-0.25	-0.31/-0.07	-0.43/-0.31	-1.42/-0.94

^aLR values are for experiment 1/experiment 2 at 8, 15, 22, 31, and 36 days after inoculation and in high/low phosphate (Pi). LR exceeding significance thresholds (0.33, 0.37, or 0.34 LR for filters A, B, and C, respectively) are shown in boldface.

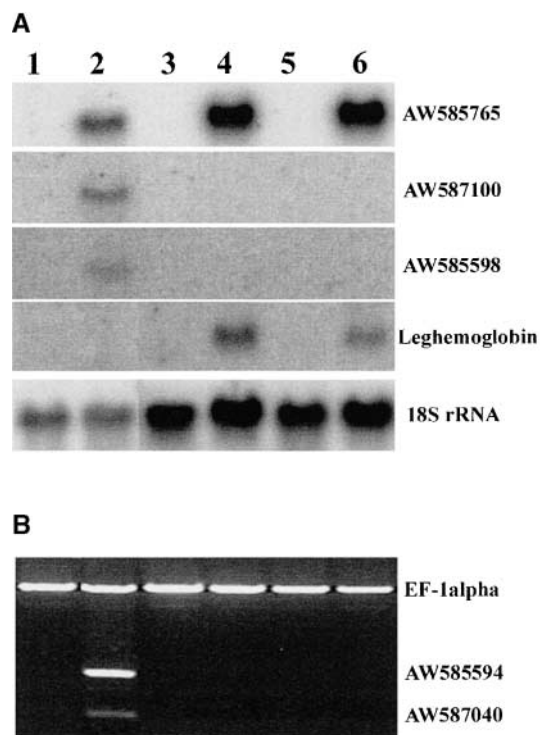


Figure 4. Expression of a Selection of the Mycorrhiza-Regulated Genes in Nodulated Roots.

(A) RNA gel blot analysis with total RNA (12 µg) from *M. truncatula* roots and *M. truncatula* roots colonized with *G. versiforme* (31 days after inoculation) (lanes 1 and 2) and *M. truncatula* roots and *M. truncatula* nodulated roots harvested at 11 (lanes 3 and 4) and 18 (lanes 5 and 6) days after inoculation. The blots were probed, stripped, and reprobbed sequentially with the probes indicated at right. The leghemoglobin gene is induced in nodules and was included as a control (Barker et al., 1988).

(B) RT-PCR analysis of the same RNA samples shown in **(A)** with primer pairs for AW585594, AW587040, and EF-1 α .

this class to be identified, is associated with expanding tissue and has been suggested to play a role in cellulose biosynthesis (Brummell et al., 1997). The Arabidopsis KOR protein is required for both cell elongation and cell plate formation and likewise is proposed to function in cell wall assembly (Nicol et al., 1998; Zuo et al., 2000). Here, we found *MtCel1* expression associated specifically with cells that contain arbuscules. Arbuscule formation is accompanied by the assembly of a new extracellular matrix in the interfacial space that develops between the arbuscule and the periarbuscular membrane. Considering the membrane domain, it is possible that *MtCel1* is located in the periarbuscular membrane and involved in the assembly of this cellulose/hemicellulose matrix. EGase gene expression has been reported widely in plant-microbe interactions, and plant EGases are induced during giant cell and syncytia formation in compatible nematode interactions (Goellner et al., 2001). In contrast to the AM symbiosis, they are all secreted EGases, which may reflect a greater requirement for degradative and remodeling activities than for biosynthetic activities.

A significant proportion of the mycorrhiza-induced genes are similar to signal transduction components. For instance, among the genes in cluster 1 are three putative transcription factors (AW584487, AW585594, and AW584152). One of these, AW585594, shows increased transcript levels in mycorrhizal roots, and based on RT-PCR analyses and the EST distribution within the gene index, expression is restricted to mycorrhizal roots. The encoded protein is similar to a group of myb/coiled-coil domain transcription factors that includes the PSR1 gene from *Chlamydomonas reinhardtii* and PHR1 from Arabidopsis (Wykoff et al., 1999; Rubio et al., 2001). PSR1 and PHR1 are required for adaptation to phosphate deprivation, and loss of function results in an inability to express a selection of phosphate starvation-response genes, including phosphate transporters, acid phosphatases, and the TPS11/Mt4 gene family. The PSR1 and PHR1 genes are expressed constitutively, but transcript levels are increased, albeit only moderately for PHR1, during phosphate starvation. By contrast, AW585594 transcripts were not regulated by phosphate but were increased coincident with the invasion of the root system by the fungus. The development of the AM symbiosis occurs most readily in phosphate-starved roots, and colonization of the root system results in the rapid, systemic, transcriptional downregulation of high-affinity phosphate transporters, acid phosphatases, and members of the TPS11/Mt4 gene family (Burleigh and Harrison, 1998; Liu et al., 1998). The mechanisms that underlie this regulation are unknown, but because the myb/coiled-coil domain factors act as dimers, heterodimerization between existing PSR1/PHR1-type proteins and a new mycorrhiza-specific partner, such as the AW585594 protein, might be one mechanism by which the expression of a large set of phosphate starvation-associated genes could be redirected.

Of the 20 mycorrhiza-induced genes in cluster 1, four are predicted to encode proteases. One of these, AW585765, shares 81% amino acid identity with a Cys protease from *As-tralagus sinicus* nodules that is proposed to play a role in the recycling of nitrogenous compounds from senescing bacteroids (Naito et al., 2000). The *M. truncatula* Cys protease is expressed in both senescing nodules and mycorrhizal roots, suggesting that the cellular processes associated with arbuscule and nodule senescence may be common to both symbioses.

There is considerable evidence for a role for proteases in plant-microbe signaling pathways, and two putative Ser carboxypeptidases (AW586622 and MtSCP1) also were expressed highly in mycorrhizal roots. Ser carboxypeptidases were identified initially in cereal grains, where they are thought to function in the degradation of storage proteins (Soerensen et al., 1987; Degan et al., 1994); however, they also have been shown to play a role in signaling pathways. Overexpression of a Ser carboxypeptidase suppressed an extracellular domain mutant of the Leu-rich repeat receptor kinase BRI1, and it was proposed that the SCP functions in the processing of a BRI1 ligand (Li et al., 2001). Our analyses indicated that the *MtSCP1* gene was expressed coordinately with the invading hyphae and displayed transient expression in the outer cortex and sustained expression in the inner cortex, with a spatial expression pattern that suggested regulation in response to a mobile signal. Given these expression patterns, a role in signaling is possible. It was

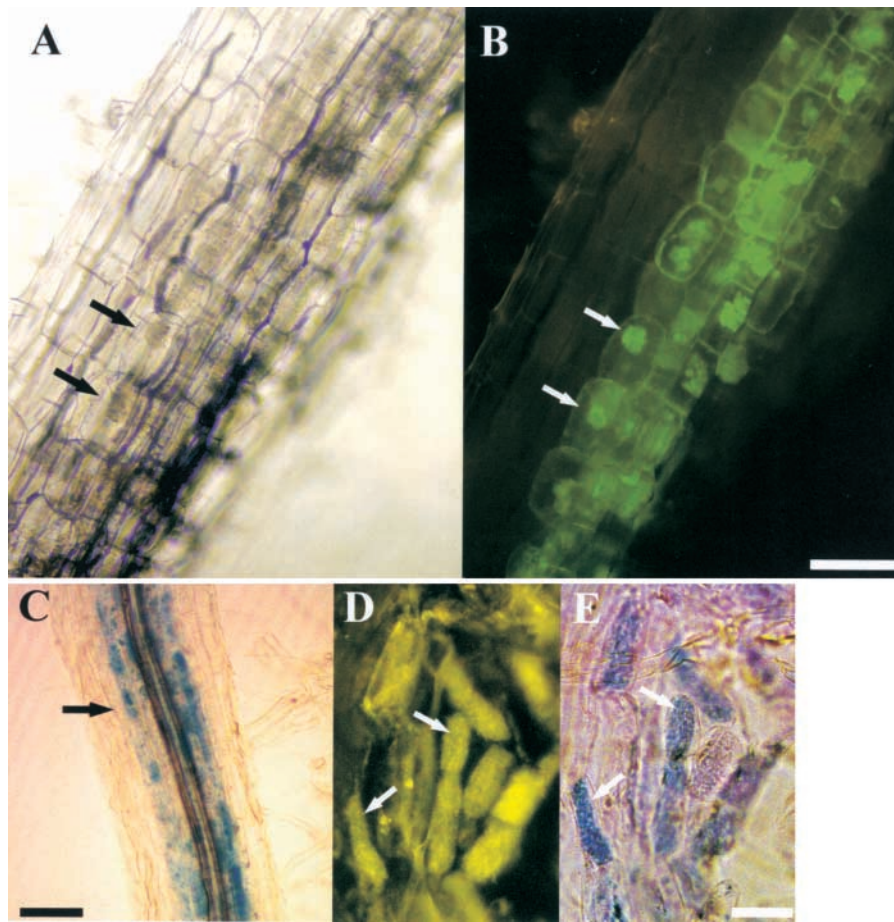


Figure 5. *M. truncatula* Roots Expressing the *uidA* and *GFP* Genes under the Control of the *MtCel1* Promoter.

(A) and **(B)** Light and corresponding epifluorescence micrographs of a *M. truncatula* root expressing *GFP* under the control of the *MtCel1* promoter. Roots were colonized with *G. versiforme*. Arrows indicate cells containing arbuscules, and *GFP* is present in cells containing arbuscules. Bar = 150 μm .

(C) Histochemical staining for GUS activity in a *M. truncatula* root expressing the *uidA* gene under the control of the *MtCel1* promoter. Arrows indicate positive staining in cortical cells containing arbuscules. Bar = 200 μm .

(D) and **(E)** Epifluorescence and corresponding light micrographs of cortical cells from a *M. truncatula* root expressing the *uidA* gene under the control of the *MtCel1* promoter. The root was stained histochemically to detect GUS activity and then counterstained with acid fuchsin to reveal the fungus. Cortical cells were released by squashing. Acid fuchsin fluoresces yellow when viewed via epifluorescence microscopy. Arrows indicate positive GUS staining in cells containing arbuscules. Bar = 40 μm .

shown recently that a Leu-rich repeat kinase, NORK/SYMRK, is required for both the development of the mycorrhizal symbiosis and nodulation (Endre et al., 2002; Stracke et al., 2002), and the NORK gene is expressed constitutively in mycorrhizal roots (L.A. Blaylock and M.J. Harrison, unpublished data). The other components of this signaling pathway are not known; however, by analogy with BRI1, MtSCP1 might be involved in ligand processing and could provide a mechanism by which the NORK/SYMRK kinase could transmit a mycorrhiza-specific signal.

One other possibility that should not be discounted is that the Ser carboxypeptidase-like proteins may not be proteases but rather acyltransferases (Lehfeldt et al., 2000; Li and Steffens, 2000; Steffens, 2000). The catalytic triad typical of the Ser carboxypeptidases is found in a wide range of enzymes, and Ser

carboxypeptidase-like proteins were shown recently to function in the transesterification of secondary metabolites. Although the enzymatic role of these proteins requires further verification, the specific spatial and temporal expression patterns support a role in the development of the symbiosis.

In conclusion, transcriptional profiling succeeded in associating a set of previously unknown genes with the development of the AM symbiosis and provided insights into the molecular response that occurs during the development of the symbiosis and clues to the specific players involved. This approach, coupled with spatial expression information, has offered a glimpse of the complexity of transcriptional changes and the signaling that occurs during the development of the AM symbiosis and provides a blueprint for future functional analyses.

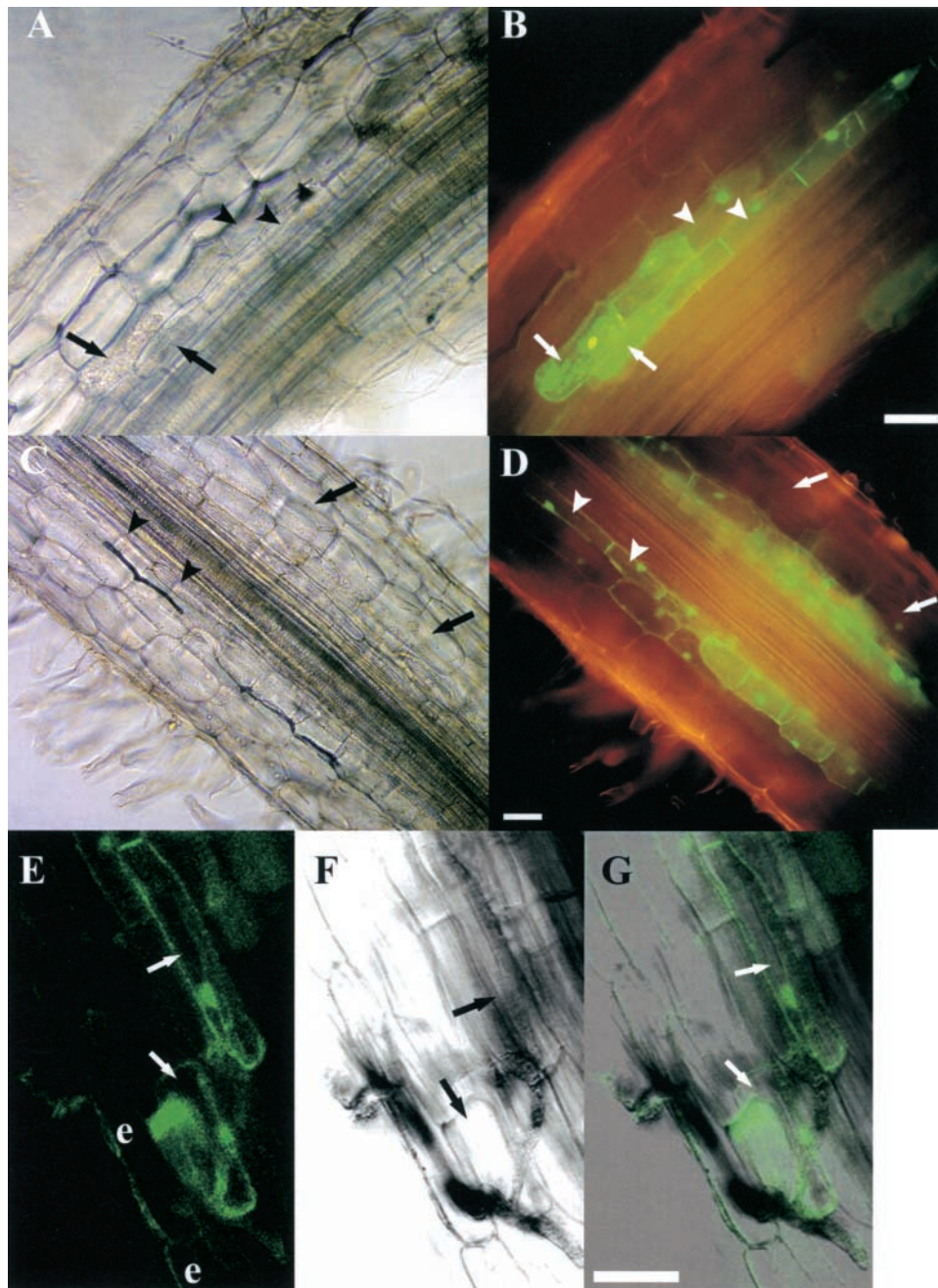


Figure 6. *M. truncatula* Roots Expressing GFP under the Control of the *MtSCP1* Promoter.

Roots were colonized with *G. versiforme*.

(A) and **(B)** Light and corresponding epifluorescence micrographs. Arrows indicate cells containing arbuscules, and a strong green fluorescence signal is present in these cells. Arrowheads indicate cells within the same cell file that do not contain arbuscules but show strong green fluorescence. Bar = 40 μm .

(C) and **(D)** Light and corresponding epifluorescence micrographs. Arrowheads indicate cells in the inner cortex that do not contain arbuscules but display strong green fluorescence. Cells containing arbuscules are present but are not in the field of focus. Arrows indicate cells in the outer cortex that show weak green fluorescence. Hyphae, but not arbuscules, are present in these cells. Bar = 40 μm .

(E) and **(F)** A laser scanning confocal microscopy image and the corresponding bright-field image of a hypha traversing the epidermis (e) and outer cortical cells. Arrows indicate outer cortical cells penetrated by the hypha, and green fluorescence is visible in these cells. Green fluorescence is not visible in the epidermal cell.

(G) Merged image showing both green fluorescence and bright-field views. Bar = 75 μm .

METHODS

Plant Materials and Growth Conditions

Medicago truncatula cv Jemalong, line A17, was used throughout this work. Plants were grown in growth rooms under a 16-h-light/8-h-dark (25/22°C) regime. The growth rooms contained F4012/Triten 50 light bulbs (www.lightbulbs4sale.com), and the light intensity at shelf height was 260 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

The growth and mycorrhizal colonization procedures were as described previously (Harrison and Dixon, 1993; Harrison et al., 2002). Briefly, 2-week-old *M. truncatula* seedlings were transplanted to sterile Turface (seven plants per 11-cm pot) and inoculated with 5000 surface-sterilized *Glomus versiforme* spores. Control plants were mock-inoculated with the final, distilled-water wash from the sterilization procedure. The plants were fertilized twice weekly with half-strength Hoagland solution (Arnon and Hoagland, 1940) containing 0.02 mM KH_2PO_4 . Plants were harvested at 8, 15, 22, 31, and 36 days after inoculation. The root systems of the seven plants from one pot were pooled, and a random sample was assessed for colonization as described previously (McGonigle et al., 1990). The remaining tissues were frozen immediately in liquid N_2 and stored at -80°C for RNA isolation and phosphate content analysis. The colonization levels of the two independent replicate experiments are listed in Table 1.

For the phosphate experiments, 10-day-old *M. truncatula* seedlings (nine plants per 11-cm pot) were transplanted to acid-washed, sterilized river sand and fertilized twice weekly with half-strength Hoagland solution containing 0.001, 0.02, 0.2, or 2.0 mM KH_2PO_4 . Levels of potassium were adjusted by the addition of the appropriate amounts of K_2SO_4 and KNO_3 . Root and shoot materials were harvested 31 or 42 days later, immediately frozen in liquid N_2 , and stored at -80°C for RNA isolation and phosphate content analysis. The phosphate content of the leaves grown under low- and high-phosphate conditions were 7.3 ± 0.4 and 31.4 ± 1.5 nmol/mg fresh weight for experiment 1 and 6.6 ± 0.3 and 28.2 ± 1.0 nmol/mg fresh weight for experiment 2, respectively.

For experiments involving nodulation, 11-day-old *M. truncatula* seedlings were transplanted to sterile Turface (eight plants per 11-cm pot) and inoculated with *Sinorhizobium meliloti*, strain 2011. The plants were fertilized twice weekly with fertilizer containing 1 mM KNO_3 and harvested at 11 and 18 days after inoculation. White nodules were visible at 11 days after inoculation, and pink nodules were clearly visible at 18 days after inoculation.

RNA Isolation and cDNA Library Construction

The *M. truncatula*/*G. versiforme* mycorrhizal cDNA library was created from RNA prepared from a pooled root sample composed of 0.70, 1.50, 2.25, 3.36, and 3.50 g of root tissue from mycorrhizal roots harvested at 10, 17, 22, 31, and 38 days after inoculation, respectively. Total RNA was extracted according to Chomczynski and Sacchi (1987), and poly(A)⁺-enriched RNA was prepared using the Oligotex mRNA Midi Kit (Qiagen, Valencia, CA). cDNA was prepared using a ZAP-cDNA synthesis kit, directionally ligated to λ ZAP II, and packaged using Gigapack III Gold packaging extracts (Stratagene, La Jolla, CA). Plasmids containing cDNA inserts were excised using Ex-Assist helper phage and propagated in SOLR cells according to the manufacturer's instructions (Stratagene). ESTs were generated by 5' end sequencing and submitted to GenBank. The ESTs also are available at the *M. truncatula* Gene Index developed at TIGR (<http://www.tigr.org/tdb/mtgi/>). The library designation is MHAM, and the library catalog number used in the gene index is T1682.

Preparation of the cDNA Arrays

The cDNA inserts of 2344 unique clones selected from the MHAM cDNA library were amplified with the following primers (5'-TGTAATACGACT-

CACTATAGGGC-3' and 5'-CGCTCTAGAACTAGTGATCCC-3') in 100- μL PCR samples (0.3 μM each primer, 125 μM deoxynucleotide triphosphates, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl_2 , 1.5 units of *Taq* DNA polymerase, and 10 ng of plasmid template). The amplification program included a 4-min denaturation at 95°C and 35 cycles of 55 s at 94°C , 55 s at 60°C , and 3 min at 72°C followed by a final extension of 8 min. All of the PCR products were examined by agarose gel electrophoresis, and only the reactions that yielded one band with an approximate DNA concentration of 100 to 200 ng/ μL were selected for spotting.

A total of 2268 cDNA inserts were spotted in duplicate on GeneScreen Plus membranes in a format containing 96 blocks of 16 inserts using the High-Density Replicating Tool and a Biomek 2000 robot (Beckman, Fullerton, CA). Approximately 20 to 40 ng of DNA was applied per spot. The set of 2268 cDNAs was encompassed on three membrane filters designated A, B, and C, and each filter also contained a set of control spots. The controls included an elongation factor-1 α (EF-1 α) cDNA (AW585952) whose expression does not change during the development of the symbiosis or in response to high- or low-phosphate conditions and a fragment of the pBluescript SK- vector (the region between 1168 and 2588 bp) as a negative control. Both the EF-1 α and pBluescript fragments were spotted twice at different locations on each filter.

Additional controls included the *Mt4* cDNA, whose expression is induced in response to phosphate starvation and downregulated in response to mycorrhizal colonization (Burleigh and Harrison, 1997), isoflavone reductase, which was shown previously to be repressed during the AM symbiosis (Harrison and Dixon, 1993, 1994), a mycorrhiza-induced cDNA, AW587100 (*MtSCP1*), which was identified in a preliminary array experiment as significantly induced in response to mycorrhizal colonization, and a 439-bp fragment of the 3' untranslated region (UTR) of the AW587100 cDNA as a second mycorrhiza-induced control spot. After spotting, the filters were subjected to alkaline denaturation, neutralization, and UV light cross-linking according to standard procedures (Sambrook et al., 1989). One set of filters was hybridized with an α - ^{32}P -dATP-labeled 222-bp fragment of the pBluescript polylinker, which was created by PCR with M13 forward and reverse primers, to verify that the arrays were spotted evenly.

Probe Preparation and Hybridization of the Arrays

The arrays were hybridized with ^{32}P -labeled first-strand cDNA probes prepared from RNA from mycorrhizal roots harvested at 8, 15, 22, 31, and 36 days after inoculation, the corresponding mock-inoculated controls, and root samples from plants grown under high-phosphate (2 mM) and low-phosphate (0.02 mM) conditions (Figure 1A). The entire experiment was replicated by hybridizing a second set of identical filters with probes from a replicate set of biological materials. For the synthesis of ^{32}P -labeled first-strand cDNA probes, 1 μg of poly(A)⁺-enriched RNA was reverse transcribed with 200 units of SuperScript II reverse transcriptase (Gibco BRL) in the presence of 40 μCi of α - ^{32}P -dATP and 40 μCi of α - ^{32}P -dCTP at 37°C . The filters were hybridized in 0.5 M NaH_2PO_4 , pH 7.4, 7% SDS, and 1 mM EDTA (Church and Gilbert, 1984) for 16 to 20 h at 65°C and then washed once in $2\times$ SSPE ($1\times$ SSPE is 0.115 M NaCl, 10 mM sodium phosphate, and 1 mM EDTA, pH 7.4) and 0.2% SDS at 65°C for 20 min, once in $0.5\times$ SSPE and 0.2% SDS at 65°C for 20 min, once in $0.2\times$ SSPE and 0.1% SDS at 65°C for 20 min, and once in $0.1\times$ SSPE and 0.1% SDS at 65°C for 20 min. Filters were exposed for 2 to 4 days, scanned using a Storm 820 PhosphorImager (Molecular Dynamics/Amersham Pharmacia, Sunnyvale, CA), and quantified using ArrayVision 5.1 software (Imaging Research, Brock University, St. Catharines, Ontario, Canada).

Data Analysis

To calculate the signal intensities for each spot, a grid was overlaid on the array image. The total intensity of all pixels within each grid was de-

terminated using the volume quantitation method (ImageQuant, Piscataway, NJ). Local background around each 4×4 subarray grid was subtracted. Signals from the duplicate spots were averaged, and adjustments for differences in the labeling and hybridization were made based on the EF-1 α spots and also on the overall signal on each filter (Hardwick et al., 1999). Because the majority of the genes on the arrays are derived from *M. truncatula*, an additional correction for the amount of *M. truncatula* RNA in the samples from mycorrhizal roots (Table 1) was included. For each replicate experiment, the ratio of the average adjusted signal in mycorrhizal/nonmycorrhizal roots and high/low phosphate was calculated for each gene and expressed as a log-10 expression ratio. Data from noisy spots, in which the replicates showed a fivefold or greater difference, were discarded. Hierarchical clustering using the correlation-centered similarity metric and the complete linkage clustering option was performed using Cluster, and the data were displayed using Treeview software (<http://rana.lbl.gov/EisenSoftware.htm>) (Eisen et al., 1998).

RNA Gel Blot Analyses

M. truncatula total RNA was extracted according to Chomczynski and Sacchi (1987), and RNA gel blots were prepared and hybridized according to standard protocols (Church and Gilbert, 1984; Sambrook et al., 1989). The blots shown in Figures 3 and 4 were sequentially stripped and reprobed with the probes indicated in each figure. The probes consisted of the entire cDNA inserts from the corresponding clone except for the MtSCP1 3' UTR probe, which consisted of 439 bp of the 3' UTR region of the MtSCP1 gene. The blots were imaged and analyzed with a Storm 820 PhosphorImager (Molecular Dynamics/Amersham Pharmacia).

Estimations of the amounts of *M. truncatula* and *G. versiforme* RNA in the mycorrhizal root RNA samples were made using gene-specific Gv-ITS and Mt-ITS probes as described previously (Maldonado-Mendoza et al., 2002).

Reverse Transcriptase-Mediated PCR Analyses

Reverse transcriptase reactions were performed with 2 μ g of RNA using SuperScript II RNase H⁻ reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Five percent of the reverse transcription reaction was used as a template in a 25- μ L PCR sample. The primers used were EF-1 α forward (5'-TCTCTCTGCGGCTGAGAGTTCC-3') and reverse (5'-TTAGGTCACAAGGCAGATTGCAGG-3'), AW585594 forward (5'-GGTCCACTCGTCA-GATGTGCTC-3') and reverse (5'-TAGGCACCAAGGTGATTGGTAGGT-3'), and AW587040 forward (5'-TTTCTGATTATCATGTTGCACTCTGC-3') and reverse (5'-GTTCTTTATCGATCCTGCTCTC-3'). These primers generate products of 1430 bp (EF-1 α), 701 bp (AW585594), and 400 bp (AW587040).

Identification of MtCel1 and MtSCP1 Promoters

Genomic clones containing the MtCel1 and MtSCP1 genes and 5' proximal regions were identified from a *M. truncatula* λ FixII genomic library using the 1408-bp partial MtCel1 cDNA and the 439-bp MtSCP1 3' UTR fragment as probes. The 5' proximal sequences of the MtCel1 gene (2085 bp) and the MtSCP1 gene (1992 bp) have been deposited in GenBank. Comparisons of the promoter regions of these genes and the mycorrhiza-induced MtPT4 promoter (Harrison et al., 2002) revealed no unique sequence motifs.

Creation of the MtCel1 and MtSCP1 Promoter-Reporter Gene Fusion Constructs

PCR-generated PstI-NcoI fragments containing 2085 bp of the MtCel1 5' proximal region and 1992 bp of the MtSCP1 5' proximal region were

ligated to the same sites of the pCAMBIA3301 vector (CAMBIA, Canberra, Australia) upstream of the uidA gene. The primers used for the amplification of the MtCel1 5' proximal region were 5'-AGCAAGCTTCTGCAGAATATGCAAGGTTTGAGGTTCAAAC-3' and 5'-CGACGGATCCATGGGATTGATGAAGATTTTGTACTTCAG-3'. The primers used for the amplification of the MtSCP1 5' proximal region were 5'-AGCAAGCTTCTGCAGCAAACCTGGCCATTATAATAATAG-3' and 5'-CGACGGATCCATGGGCTTGTCTCAACTTGGTTTCAACA-3'. In both cases, this cloning strategy resulted in the insertion of 10 additional bases (CCATGGATCC) before the first ATG of the uidA gene.

For the preparation of MtCel1 and MtSCP1 promoter-sGFP fusion constructs, the same 5' regions containing HindIII-BamHI ends were ligated into the HindIII-BamHI sites of a pCAMBIA3300 vector carrying a HindIII-EcoRI fragment from the CaMV35S-sGFP(S65T)-nos plasmid (Chiu et al., 1996). Insertion of the MtCel1 or MtSCP1 5' region replaces the 35S promoter in each instance. This cloning strategy introduces two additional bases (CC) before the first ATG of the GFP gene. Binary vectors were transformed into *Agrobacterium rhizogenes* strain ARqual using standard methods.

Preparation and Analysis of M. truncatula Plants Containing Transformed Roots

M. truncatula plants with transformed roots were created and grown as described previously (Boisson-Dernier et al., 2001; Harrison et al., 2002) with the following modifications. The seedlings were grown on a modified medium (1 mM NH₄NO₃, 0.9 mM CaCl₂, 0.5 mM MgSO₄, 20 μ M KH₂PO₄, 10 μ M Na₂HPO₄, and 20 μ M ferric citrate supplemented with 100 μ g/L H₃BO₃, 33 μ g/L MnCl₂, 33 μ g/L CuSO₄, 7 μ g/L ZnCl₂, 33 μ g/L Na₂MoO₄, and 200 mg/L Mes, pH 6.4, containing 5 mg/L phosphinothricin). To prevent contact of the leaves with phosphinothricin in the medium, sterilized pieces of Parafilm were placed on the agar below the cotyledons. Plants with transgenic root systems were transplanted and inoculated with *G. versiforme* spores as described previously (Harrison et al., 2002) and examined between 6 and 33 days after inoculation. The expression of the uidA gene was evaluated by GUS staining in 0.1 M phosphate buffer, pH 7.0, containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 10 mM EDTA, 1 mg/mL 5-bromo-4-chloro-3-indolyl β -D-glucuronic acid, and 1% (v/v) N,N-dimethylformamide. Roots were counterstained with acid fuchsin to enable the localization of *G. versiforme* within the roots. GFP expression was evaluated by fluorescence microscopy. Roots were hand-sectioned and examined by light, epifluorescence, and confocal microscopy as described previously (Harrison et al., 2002). The expression patterns described were observed in a minimum of 10 independent transgenic root systems.

Determination of Phosphate Content

Freshly harvested shoot samples were frozen in liquid nitrogen, and total phosphate in ashed and hydrolyzed samples was determined by phosphomolybdate colorimetric assay (Ames, 1966).

Upon request, materials integral to the findings presented in this publication will be made available in a timely manner to all investigators on similar terms for noncommercial research purposes. To obtain materials, please contact Maria J. Harrison, mjh78@cornell.edu.

Accession Numbers

The accession numbers for the sequences mentioned in this article are as follows: MtSCP1 cDNA, AY308957; MtSCP1 5' proximal sequence, AY308958; MtCel1 cDNA, AY308955; MtCel1 5' proximal sequence, AY308956; *M. truncatula* Cys protease cDNA, AY336982; KOR,

AF073875; Cel16, CAB51903; Cel1, BAA94257; Cel3, AAC49704; and IFR, X58078.

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