Novel Genes Induced During an Arbuscular Mycorrhizal (AM) Symbiosis Formed Between *Medicago truncatula* and *Glomus versiforme*

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Many terrestrial plant species are able to form symbiotic associations with arbuscular mycorrhizal fungi. Here we have identified three cDNA clones representing genes whose expression is induced during the arbuscular mycorrhizal symbiosis formed between *Medicago truncatula* and an arbuscular mycorrhizal fungus, *Glomus versiforme*. The three clones represent *M. truncatula* genes and encode novel proteins: a xyloglucan endotransglycosylase-related protein, a putative arabinogalactan protein (AGP), and a putative homologue of the mammalian p110 subunit of initiation factor 3 (eIF3). These genes show little or no expression in *M. truncatula* roots prior to formation of the symbiosis and are significantly induced following colonization by *G. versiforme*. The genes are not induced in roots in response to increases in phosphate. This suggests that induction of expression during the symbiosis is due to the interaction with the fungus and is not a secondary effect of improved phosphate nutrition. In situ hybridization revealed that the putative AGP is expressed specifically in cortical cells containing arbuscules. The identification of two mycorrhiza-induced genes encoding proteins predicted to be involved in cell wall structure is consistent with previous electron microscopy data that indicated major alterations in the extracellular matrix of the cortical cells following colonization by mycorrhizal fungi.

Arbuscular mycorrhizae (AM) are symbiotic associations formed between fungi of the order Glomales, and the roots of many terrestrial plants (Harley and Smith 1983). These symbionts have apparently coexisted for many millions of years,

as fossil evidence indicates the presence of AM fungi in the early land plants of the Devonian era (Remy et al. 1994). Today, the symbiosis is estimated to occur in more than 80% of extant plant taxa and is an integral part of ecosystems throughout the world. The association is considered mutually beneficial; the plant supplies the fungus with carbon on which it is entirely dependent, while the fungus assists the plant with the uptake of phosphate and other mineral nutrients from the soil (reviewed in Smith and Gianinazzi-Pearson 1988). In some instances, colonization by mycorrhizal fungi also improves the plant's resistance to root pathogens (Newsham et al. 1995). The potential agronomic benefits of the association, particularly for low-input, sustainable agriculture, have been recognized (Jeffries 1987; Bethlenfalvai 1992).

The development of the functional symbiosis requires a complex series of interactions between the two symbionts. This is initiated when the fungal hyphae contact the root surface, differentiate to form an appressorium, and subsequently penetrate the root. Once inside the root, the hyphae grow inter- and intracellularly throughout the cortex and differentiate within the cortical cells to form dichotomously branched structures known as arbuscules (Bonfante-Fasolo 1984; Gianinazzi-Pearson 1996). The arbuscule/cortical cell interface is predicted to be the site at which carbon and phosphate transfer occurs (Smith and Smith 1990; Smith 1993). Immunocytochemistry and electron microscopy studies have revealed significant alterations in the cell wall and membranes of the symbionts during the symbiosis, particularly at the interfaces between the cortical cells and the developing arbuscules (Gianinazzi-Pearson et al. 1991; Bonfante-Fasolo and Perotto 1992; Bonfante and Bianciotto 1995). Although the process of colonization has been clearly described, the molecular mechanisms underlying the development of the symbiosis and the signaling between the two symbionts are still largely unknown.

Genetic and molecular investigations are providing new insight into the symbiosis (reviewed in Harrison 1997). Plant mutants unable to form a complete AM symbiosis have been identified, indicating that development of the association is controlled, at least in part, by the plant (Duc et al. 1989; Bradbury et al. 1991; Sagan et al. 1995). These mutants were all identified from populations of nodulation mutants and, as the phenotypes are tightly linked, it seems likely that there are

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Nucleotide and/or amino acid sequence data will appear in the GenBank and EMBL data bases under the following accession numbers: Mt.AM1, AF106929; Mt.AM2, AF093506; Mt.AM3-1, AF106930; and Mt.AM3-2, AF106931.

common genes whose expression is essential for both symbioses. The induction of some of the nodulin genes in mycorrhizal roots is a further indication of similarities in the molecular events occurring in these symbioses (Frühling et al. 1997; van Rhijn et al. 1997).

To date, molecular studies on gene expression in AM associations have focused mainly on previously identified plant genes induced during plant-pathogen interactions. In general, the defense genes show only minor transient increases, or decreases in expression in mycorrhizal roots, which is significantly different from expression patterns observed during pathogenic interactions (Harrison and Dixon 1993; Lambais and Mehdy 1993; Franken and Gnädinger 1994; Harrison and Dixon 1994; Volpin et al. 1995). Four cDNAs representing non-defense-related plant genes whose expression is increased in mycorrhizal roots have been reported: a sugar transporter from *Medicago truncatula* (Harrison 1996); a putative membrane protein of unknown function (Martin-Laurent et al. 1997); a membrane intrinsic protein (MIP) gene from parsley (Roussel et al. 1997); and an ATPase from *Hordeum vulgare* (Murphy et al. 1997). An additional cDNA encoding a protein of unknown function has also been reported (Murphy et al. 1997).

The isolation of genes induced during the AM symbiosis is a prerequisite for understanding the molecular basis of its development and regulation. Here we describe the identification and characterization of three novel plant cDNAs representing genes induced in the mycorrhizal symbiosis. One of the cDNAs encodes a putative arabinogalactan protein (AGP) whose expression is localized exclusively in cortical cells containing arbuscules.

RESULTS

Isolation of cDNA clones and expression in roots colonized by a mycorrhizal fungus.

A cDNA library with 2.5-fold enrichment for mycorrhiza-specific sequences was prepared by subtractive hybridization and differentially screened with ^{32}P -labeled cDNA from non-colonized (control) (Mt) and colonized roots (Mt/Gv). Forty-five cDNA clones were selected and further analyzed by Northern (RNA) blot hybridization. Three clones, Mt.AM1, Mt.AM2, and Mt.AM3, were identified that represent genes that show increased expression in colonized roots. Of the remaining cDNA clones, 12 represent genes with transcript levels too low to be detected by Northern blot analysis and 28 represent genes that show similar transcript levels in control and colonized roots.

The appearance of the Mt.AM1, Mt.AM2, and Mt.AM3 transcripts during the development of the mycorrhizal association was further investigated in a time course experiment in which *M. truncatula* plants were inoculated with *Glomus versiforme* spores and harvested at weekly intervals up to 44 days post inoculation (dpi). The mycorrhizal time course is not a synchronous system; however, broadly overlapping stages can be distinguished (Harrison and Dixon 1993). In this time course, the earliest time point (8 dpi) features predominantly surface growth of fungal hyphae and appressoria formation, with very limited internal colonization. By 15 dpi, internal colonization is progressing rapidly. At 22 and 29 dpi, the roots are highly colonized (in the region of 57 and 71% root length

colonized, respectively) and all of the fungal structures are present, but arbuscules and internal hyphae predominate. By the final time point (44 dpi) arbuscules, internal hyphae, and vesicles are all apparent. Northern blots containing root RNA from the time course were hybridized with the cDNA inserts from the 3 clones (Fig. 1). Mt.AM1 hybridizes to a 0.9-kb transcript present at low or undetectable levels in control roots and induced in colonized roots. The transcript is present at 8 dpi, reaches a maximum level at 15 dpi, and remains at an elevated level throughout the remainder of the time course (Fig. 1A). Mt.AM3 hybridizes to two transcripts, one of approximately 3 kb that is constitutively expressed in control and colonized *M. truncatula* roots, and a second of 1.4 kb that is induced in colonized roots. The 1.4-kb transcript is present at 8 dpi and reaches maximum levels at 29 dpi (Fig. 1A). Mt.AM2 hybridizes to a transcript of approximately 1.3 kb that is present exclusively in the colonized roots. The transcript is first detected at 22 dpi and the levels increase, reaching a maximum at 44 dpi. The Mt.AM2 transcript level is exceedingly low and the prolonged exposure necessary to detect this transcript resulted in the appearance of background hybridization to two constitutive bands assumed to be rRNAs (Fig. 1B).

The mycorrhizal symbiosis frequently results in increases in the phosphate status of the plant (Smith and Read 1997). Therefore a gene whose expression is regulated during devel-

Fig. 1. Expression of Mt.AM1, Mt.AM2, and Mt.AM3 in *Medicago truncatula* roots colonized with *Glomus versiforme*. Northern (RNA) blot analysis of RNA from roots of *M. truncatula* that had been mock inoculated (Mt) or inoculated with *G. versiforme* (Mt/Gv) at day 0 and harvested at 8, 15, 22, 29, and 44 days post inoculation. Blots were hybridized with Mt.AM1, Mt.AM2, Mt.AM3, and pSR1-2B3 (18S rRNA) as indicated. Arrows denote induced transcripts. Induction of these transcripts has also been observed in either a second time course, or in two additional mycorrhizal samples.

opment of the association may be responding directly to fungal colonization or, alternatively, to increases in the phosphate content of the plant. To determine whether the Mt.AM1, Mt.AM2, and Mt.AM3 transcripts show regulation in response to phosphate nutrition, the transcript levels were examined in root RNA from plants grown under different phosphate regimes (Liu et al. 1998). In all three cases there was no induction of these transcripts in response to increasing phosphate levels (Fig. 2). To act as a positive control, the same Northern blot was stripped and reprobed with a phosphate starvation-inducible *M. truncatula* phosphate transporter cDNA (MtPT1) (Liu et al. 1998). The MtPT1 gene showed high levels of expression in the phosphate-deficient roots (0 mM), indicating that these plant were deprived of phosphate. Transcript levels were lower in the phosphate-sufficient plants (1 mM). The slight increase in the level of MtPT1 transcripts in the 0.1 mM treatment relative to the 0.02 mM treatment is probably due to unequal loading, as indicated by the rRNA controls. The phosphate content of these plants has been reported previously and is positively correlated with the level of phosphate fertilization (Liu et al. 1998).

Genomic origin of the cDNA clones and gene organization.

The cDNA clones were isolated from a mycorrhizal library that contains representatives of both the plant and fungal genomes and therefore it was necessary to determine their origin. As the mycorrhizal fungi are obligate symbionts it is difficult to obtain sufficient pure DNA for Southern blots and so the origin of the clones was determined by PCR (polymerase chain reaction). A PCR approach also has the advantage of greater specificity since highly conserved genes may show cross hybridization between genomes in Southern analysis. Primers were designed to the individual cDNA clones and used in PCR amplifications to amplify the corresponding DNA fragments from *M. truncatula* or *G. versiforme* genomic

DNA. Primers corresponding to Mt.AM1, Mt.AM2, and Mt.AM3 were able to amplify DNA fragments from the cDNA control plasmids and from *M. truncatula* genomic DNA but did not amplify DNA fragments from *G. versiforme* genomic DNA. The amplified fragments hybridized with the appropriate cDNA probes (Fig. 3). A *G. versiforme* cDNA (Gv1) was included as a positive control for amplification from *G. versiforme* DNA. Primers corresponding to Gv1 amplified the appropriate DNA fragment from *G. versiforme* genomic DNA. In this case, the DNA fragment amplified from the genomic DNA is larger than that amplified from the cDNA control due to the presence of an intron (Fig. 3) (Burleigh and Harrison 1998).

Southern blot analyses of *M. truncatula* genomic DNA revealed that Mt.AM1 and Mt.AM3 represent members of multigene families (Fig. 4A and C). The Mt.AM1 cDNA contains a single *Hind*III site but no *Eco*RI, *Eco*RV, or *Bam*HI sites and therefore the gene family can be predicted to be fairly large. Mt.AM3 contains a single *Hind*III site, three *Eco*RI sites, and an *Eco*RV site, and therefore the family may contain two or possibly three members. Mt.AM2 does not contain any of the restriction sites used in the Southern analysis and hybridizes to a single high-molecular-weight fragment that probably represents a single gene, although the possibility of additional genes on this fragment cannot be ruled out (Fig. 4B).

Characterization of cDNA clones.

The Mt.AM1 cDNA is 912 bp and contains a complete open reading frame that is predicted to encode a protein of 191 amino acids. A subsequent screen with the Mt.AM1 cDNA as a probe resulted in the identification of three additional cDNA clones identical in sequence to Mt.AM1. Sequence analysis revealed that Mt.AM1 is novel and does not share significant similarity with any sequences in the data bases; however, the amino acid sequence contains some motifs typical of the protein backbone of a class of highly glycosylated cell wall pro-

Fig. 2. Mt.AM1, Mt.AM2, and Mt.AM3 are not induced in response to growth under different phosphate regimes. Northern (RNA) blot of root RNA from *Medicago truncatula* plants fertilized with one-half Hoagland's solution containing 0, 0.02, 0.1, and 1.0 mM phosphate. Blots were hybridized with Mt.AM1, Mt.AM2, Mt.AM3, *M. truncatula* phosphate transporter (MtPT1), and pSR1-2B3 (18S rRNA) as indicated.

Fig. 3. Southern blot of PCR (polymerase chain reaction)-amplified products from *Medicago truncatula* and *Glomus versiforme* genomic DNAs. Primer combinations specific for Mt.AM1, Mt.AM2, Mt.AM3, and a known *G. versiforme* gene, Gv1, were used to amplify the corresponding genes from samples of *M. truncatula* genomic DNA (Mt) and *G. versiforme* genomic DNA (Gv). The appropriate cDNA clone was included as a control (c) for each PCR. PCR products were separated by gel electrophoresis, blotted, and hybridized with the individual cDNAs.

teins termed AGPs (Du et al. 1996a). The deduced Mt.AM1 protein contains a potential N-terminal signal sequence that suggests that it is transported out of the cell (Fig. 5). Following the rules of von Heijne (1986), a cleavage site is predicted between the alanine and glutamine residues at positions 23 and 24, resulting in a mature protein of 168 amino acids with a molecular mass of 14.9 kDa. The mature protein starts with a short 27 amino acid domain containing a very high percentage (52%) of the proline residues. Unlike the remainder of the protein, the proline residues in this region are not interspersed with other residues but are clustered (Fig. 5). Two such clusters of proline residues match consensus sequences for the Src-homology 3 (SH-3) ligands that have been identified previously in a diverse array of mammalian and yeast proteins (Ren et al. 1993). SH-3 ligands bind SH-3 domains, which are regions with sequence similarity to the c-src proto-oncogene protein. The ligand and the SH-3 domains mediate protein-protein interactions and are often found in signaling proteins. The amino acid sequence APGTPPPAEP, found between residues 32 and 41, matches exactly the consensus binding sequence for the c-abl SH3 domain (Freeman et al. 1996). Following this proline-rich region is a hydrophilic domain with a high percentage (43%) of lysine and aspartic acid residues that occur as a short repeat sequence "KGKDT/A" (Fig. 5). The third domain of the mature protein, beginning with amino acid 79, is 73 amino acids long and rich in alanine (31%), proline (22%), glycine (21%), and threonine (12%). Despite the predominance of glycine and proline residues, the repeat motifs that are typically found in the cell wall proline-rich proteins

(PRPs) or glycine-rich proteins (GRPs) are not present (Showalter 1993). The only repeat is AAPG/S/T, which occurs four times. Based on the rules of Englund (1993), the C-terminal end of the protein contains a putative signal sequence for the addition of a glycosyl phosphatidylinositol (GPI) anchor, a hydrophobic domain of 17 amino acids preceded by a hydrophilic region, and a group of three small amino acids. The anchor is predicted to be added to the glycine at position 168 and the cleavage site is predicted to be between the glycine at position 168 and alanine at position 169 (Englund 1993). GPI anchors are glycolipids that are covalently attached to proteins and anchor them to the external face of the membrane. They are widespread in mammals and have been described recently in plants (Takos et al. 1997).

The initial Mt.AM2 clone contained a cDNA insert of 548 bp and data from both Northern blot and sequence analyses indicated that it was a truncated cDNA clone. This sequence was used to obtain a genomic clone (I. E. Maldonado-Mendoza and M. J. Harrison, *unpublished data*), which then enabled the amplification and cloning, by reverse transcriptase (RT)-PCR, of a full-length cDNA from RNA from mycorrhizal roots. The cDNA (Mt.AM2) is 1,107 bp in length and is predicted to encode a protein of 292 amino acids that shares significant sequence identity with xyloglucan endotransglycosylase (XET) and XET-related sequences (XTR) from a variety of plant species. XETs are enzymes that modify xyloglucan polymers in the cell wall by cleaving and rejoining the individual xyloglucan molecules (Smith and Fry 1991; Nishitani and Tominaga 1992). The 292 amino acid open reading frame from Mt.AM2 is most similar to an XTR from cotton

Fig. 4. Southern blot analysis of *Medicago truncatula* genomic DNA hybridized with (A) Mt.AM1, (B) Mt.AM2, and (C) Mt.AM3. DNA was digested with *Bam*HI (B), *Eco*RI (E1), *Eco*RV (E2), and *Hind*III (H). The size markers (kb) are shown to the right.

Fig. 5. DNA sequence and deduced amino acid sequence of Mt.AM1. Boxed sequence is a potential signal sequence; underlined sequence is the proline-rich ('SH3 type') domain; KGKDA pentapeptide repeat is shown in bold; dashed, underlined sequence represents the alanine, proline, glycine, threonine-rich domain. Dashed, boxed sequence is a hydrophobic domain considered to be part of the putative GPI anchor signal sequence. Putative GPI anchor site is indicated by a vertical line.

(*Gossypium hirsutum*) (D88413; Shimizu et al. 1997) with which it shares 74% amino acid identity and 89% similarity (Fig. 6). The predicted Mt.AM2 protein also contains a number of features commonly found in XET and XTR proteins including a putative N-terminal signal sequence and potential N-linked glycosylation sites following a conserved sequence DEIDFELFG that has been proposed to be within the active site critical for the cleavage of (1-4) β -glycosyl linkages (Borriss et al. 1990; Okazawa et al. 1993; Xu et al. 1996). Four cysteine residues, which are highly conserved in the C-terminal portion of XET and XTR proteins and are thought to be important for the formation of disulfide bridges, are also found in the Mt.AM2 protein (Okazawa et al. 1993).

Mt.AM3 contains a cDNA insert of 557 bp including a poly A tail at the 3' end. This cDNA was subsequently used as a probe to re-screen the mycorrhizal cDNA library, resulting in the isolation of two additional clones, Mt.AM3-1 and Mt.AM3-2, that contain cDNA inserts of 3,246 and 1,407 bp, respectively. Mt.AM3-2 represents the same gene as the original Mt.AM3 cDNA whereas Mt.AM3-1 is slightly different. Within the 1,407 bp of overlapping sequence Mt.AM3-1 and Mt.AM3-2 share 94% nucleic acid and amino acid identity and probably represent two different members of the multi-gene family. Mt.AM3-1 contains a complete open reading frame and is predicted to encode a protein of 935 amino acids. Data base searches reveal that Mt.AM3-1 shares 33% amino acid identity with the P110 subunit of the eukaryotic translation initiation factor 3 complex (eIF3-p110), which was recently cloned from humans (Fig. 7) (Asano et al. 1997) and 65% identity with an *Arabidopsis thaliana* EST (AF040102) that also shares sequence similarity with human eIF3-p110. The Mt.AM3-2 cDNA contains an AUG start codon 4 nucleotides from the 5' end of the clone and the predicted open reading frame contains 383 amino acids and shares 39% amino acid identity with the 3' half of eIF3-p110. The original Mt.AM3 clone hybridized to two transcripts on the Northern blots: a constitutive transcript of approximately 3 kb and an inducible 1.4-kb transcript (Fig. 1A). Mt.AM3-1 clearly represents the 3-kb transcript and Mt.AM3-2 may represent the 1.4-kb transcript; however, the possibility that it represents a truncated version of a longer transcript cannot be excluded.

Localization of Mt.AM1 transcripts in *M. truncatula* roots colonized with *G. versiforme*.

In situ hybridization was used to obtain further insight into the expression of Mt.AM1 in mycorrhizal roots. For technical reasons, Mt.AM2 and Mt.AM3-2 were not further analyzed by this approach. Mt.AM2 transcripts are present at extremely low levels and Mt.AM3-2 cross hybridizes with a constitutively expressed transcript, Mt.AM3-1, making it difficult to interpret the results. The location of the expression of these genes would be more readily analyzed by transgenic approaches.

Whole mount in situ hybridization was carried out on 2- to 4-mm root pieces from a *M. truncatula* root system colonized with *G. versiforme*. Hybridization of the root pieces with an antisense Mt.AM1 probe revealed the presence of Mt.AM1 transcripts, indicated by purple/blue staining, specifically in cortical cells containing arbuscules (Fig. 8A). Root pieces hybridized with the sense control probe did not show any staining in the cortical cells containing arbuscules. In this case, the

arbuscules can be seen within the cells as diffuse, slightly yellow, hyaline structures (Fig. 8B). The same result was obtained by hybridization of probes to thin sections of mycorrhizal roots (Fig. 8C and D). Figure 8C shows an 8- μ m transverse section through mycorrhizal root hybridized with an Mt.AM1 antisense probe. The Mt.AM1 transcripts, indicated by the presence of purple/blue staining, are present in those inner cortical cells that contain arbuscules. A serial section hybridized with a sense Mt.AM1 probe shows a considerably lower level of staining in cells containing arbuscules (Fig. 4D). This staining, and also the limited amount of staining of the xylem tissue regardless of the probe, are interpreted as nonspecific background staining. The cells of the xylem have fairly thick cell walls and the arbuscules have a very large surface area of wall-like material, both of which cause them to bind a low level of the colored precipitate nonspecifically. Consistent with the Northern blot data, *M. truncatula* roots that were not colonized did not show any staining in any part of the root, including root tips and emerging laterals (data not shown).

DISCUSSION

The identification of genes induced specifically in plant roots during their interactions with rhizobia (Franssen et al. 1987; Govers et al. 1987; Scheres et al. 1990a, 1990b) or nematodes (Gurr et al. 1991; Opperman et al. 1994) provided initial information about the molecular responses to these organisms and served as a starting point for subsequent molecular and genetic analyses of the interactions. Despite the widespread occurrence of the AM symbiosis our current understanding of the molecular events underlying its development lags behind that of the other major root/microbe interactions. The three plant cDNAs identified here begin to address this and provide molecular genetic evidence that supports alterations in the extracellular matrix and in components of the translational machinery following formation of the mycorrhizal symbiosis.

Mt. AM2	1- MAITSPFFKHNVSFFILLMILLSCVF-----VWGRPATFNQDFHVTWSEP	-45
Gh. XET	1- LILSCVTVLTLVLGRFATFLDFRITWSDS	-31
Mt. AM2	46- HIKQIDQRTIQLTLNQSCGCFASKVKYLFGRVSMKILVPGDSAGTIV	-95
Gh. XET	32- HIRQIDGGRATQLVLDQNSGCFASKRYLFGRVSMKILIPGDSAGTIV	-81
Mt. AM2	96- AFYMNSDTSVRDELDFEFLGNRTGQPYTVQNTIYAHGKGDREQRVNLWF	-145
Gh. XET	82- AFYMNSVTDAVRDELDFEFLGNRTGQPYTVQNTIYAHGKGDREQRVNLWF	-131
Mt. AM2	146- DPSADPHTYSILWNHGHIVSHVDEVPVIRVYKNEAKGIPYPMQAMGVFS	-195
Gh. XET	132- DPAADPHTYSIMWNGHIVFYIDEVPVIRVYKNEARNIPYPMQAMGVFS	-181
Mt. AM2	196- TLWEADNWATRGGLEKINWSKAPFYAYKDFDIEGCAIPGPTCTSTNPKN	-245
Gh. XET	182- TLWEADNWATRGGLEKIDWTKAPFLAYKDFDIEGCPVPGPVNCAATNSRN	-231
Mt. AM2	246- WVEGVBYQALSAIEARRYRWVNMNHYDYCDKSRYPWTPHECLSGI	-293
Gh. XET	232- WVEGTAYQALNAMEAKRYRWVNMNHYDYCDKSRYPVTPPECMSII	-279

Fig. 6. Alignment of predicted amino acid sequence of Mt.AM2 with the *Gossypium hirsutum* xyloglucan endotransglycosylase (XET) (D88413) amino acid sequence. '/' indicates identical amino acids; '.' indicates similar amino acids. Putative signal peptide sequence is in bold; conserved region suggested to be the active site is outlined with a box. Putative glycosylation sites are outlined with a dashed box. Conserved C terminal cysteine residues are shaded.

The AGPs are a heterogeneous family of highly glycosylated, cell wall proteins that have been found in a wide array of plant tissues and secretions (Clarke et al. 1979; Showalter 1993; Du et al. 1996a). The protein backbones of several AGPs have been cloned and although the primary sequences

The precise functions of the AGP-type proteins are unclear; however, there is evidence for their involvement in a diverse array of cellular processes including cell proliferation, expansion, differentiation, somatic embryogenesis, and pollen tube growth (Du et al. 1994, 1996b; Kreuger and van Holst 1996; Willats and Knox 1996; Langan and Nothnagel 1997). AGP-like proteins have also been reported in several plant symbiotic associations. *Enod 5*, a gene encoding an AGP-like protein, is induced during nodule development in the *Rhizobium* spp.-legume symbiosis (Scheres et al. 1990b). AGPs have also been detected in the *Gunnera* spp. stem gland mucilage, which is central for communication between *Gunnera* spp. and their cyanobacterial symbiont *Nostoc* spp. (Rasmussen et al. 1996). In the *M. truncatula*/*G. versiforme* symbiosis, Mt.AM1 expression is induced exclusively in the cells containing arbuscules. This location coupled with its predicted identity as an AGP is consistent with earlier immunological data that demonstrated the presence of arabinosylated β -(1,6)-galactan epitopes in the host periarbuscular membrane that surrounds the arbuscule and also with the contents of the interface compartment (Perotto et al. 1994; Gollotte et al. 1995; Balestrini et al. 1996). We speculate that Mt.AM1 may be a structural component of the interface compartment or, alternatively, by analogy to the pollen tube system, it might be involved in mediating the interaction between the plant cortical cell and fungal hypha during arbuscule development. As the expression is localized specifically to the cells containing arbuscule and not to adjacent cortical cells, it suggests that the signals inducing this expression are cell autonomous.

[illegible]

of the cortical cell walls. The observation that the Mt.AM2 transcript levels are low and are only detected when considerable colonization of the root has occurred is consistent with these suggestions.

The other clones identified in the approach (Mt.AM3-1 and Mt.AM3-2) share sequence similarity with the P110 subunit gene of the human translation initiation factor 3 (eIF3-p110). eIF3 is a large, multi-subunit complex, consisting of at least eight polypeptides, that binds to the 40S ribosome early in translation initiation and stabilizes the binding of methionyl-

tRNA and mRNA (Asano et al. 1997). Initiation of protein synthesis is one site of translational control and, in yeast, both eIF2 and eIF3 are implicated in this process (Hannig 1995). Based on size and sequence identity it seems likely that Mt.AM3-1 represents an eIF3-P110 homologue. The function of the product of the 1.4-kb inducible transcript is currently unclear. It is probably too small to be a functional homologue of the P110 gene but might have another role in translation initiation. Mycorrhiza-specific, eIF3-like transcripts can be detected early in the development of the association and might

Fig. 8. Localization of Mt.AM1 transcripts in *Medicago truncatula* roots colonized with *Glomus versiforme*. **A**, Whole mount in situ hybridization of a root piece hybridized with an antisense Mt.AM1 probe. A positive signal indicated by dark purple staining is present in the cortical cells containing arbuscules. Diffuse images of the arbuscules are visible within these cells (indicated by arrows). Scale bar = 38 µm. **B**, Whole mount in situ hybridization of a root piece hybridized with a sense Mt.AM1 probe. Diffuse, slightly yellow shapes of the arbuscules can be seen in the inner cortical cells (indicated by arrows). Scale bar = 53 µm. **C** and **D**, Transverse sections (8 µm) through an *M. truncatula* root colonized with *G. versiforme* hybridized with antisense and sense Mt.AM1 probes, respectively. Sections are serial sections not more than 24 µm apart. **C**, Positive signal indicated by purple staining is present in cortical cells containing arbuscules. Scale bar = 60 µm. Cell and tissue types: c, cortex; a, cortical cell containing an arbuscule; v, vascular cylinder.

indicate that translational control plays a role in regulating de novo protein biosynthesis during the formation of mycorrhizae. Down-regulation of a group of phosphate-starvation-inducible genes has been observed following colonization (Burleigh and Harrison 1997; Liu et al. 1998). It is possible that a switch in the translational machinery leads to their exclusion from the translational machinery and accelerates their subsequent degradation.

The isolation of cDNA clones differentially expressed in mycorrhizae is the first stage in unraveling the molecular processes involved in this complex symbiosis. Here we have identified three plant genes whose expression is significantly induced following colonization by a mycorrhizal fungus. In addition to providing initial insight into the molecular changes that occur in plant roots during the development of the symbiosis, these cDNA clones are essential tools for future analyses of the symbiosis. The corresponding mycorrhizal-inducible promoters will provide a starting point for the analysis of the signal transduction pathways between the two symbionts.

MATERIALS AND METHODS

Stock cultures of AM fungi.

The preparation and maintenance of stock cultures of *G. versiforme* have been described previously (Harrison and Dixon 1993). Briefly, spores were collected and surface sterilized in freshly prepared sterilizing solution (2% [wt/vol] chloramine T, 200 µg of streptomycin per ml, 0.01% [vol/vol] Tween 20) for 20 min. Spores were subsequently washed five times in sterile, distilled water and used to inoculate plants. The water from the final wash was used to inoculate control plants.

Plant material.

M. truncatula cv. Jemalong was grown and colonized with *G. versiforme* as previously described (Harrison and Dixon 1993).

In the time course experiment, roots of 2-week-old seedlings (7 to 10 plants per pot) were inoculated with 5,000 spores from *G. versiforme* sporocarps. Control plants were mock inoculated with the final distilled water wash from the sterilization procedure. Plants were harvested 8, 15, 22, 29, and 44 dpi and colonization levels assessed as described previously (Harrison and Dixon 1993). Colonization levels were in the region of 12, 20, 57, 71, and 72% root length colonized, respectively.

The RNA samples from plants grown with increasing levels of phosphate fertilization were the same as those used previously (Liu et al. 1998). Briefly, *M. truncatula* seedlings were grown for a further 46 days in sterilized sand and fertilized three times a week with 1/2 strength Hoagland's solution containing either 0, 0.02, 0.1, or 1.0 mM KH_2PO_4 . The levels of potassium were maintained by addition of the appropriate amount of K_2SO_4 . The phosphate levels in the leaves of plants were 3.96×10^{-3} , 8.65×10^{-3} , 17.03×10^{-3} , and 29.75×10^{-3} µmol per mg of dry tissue for the 0, 0.02, 0.1, and 1.0 mM KH_2PO_4 samples, respectively (Liu et al. 1998).

Nucleic acid extraction and blot analysis.

RNA was extracted from root tissue as described by Chromczynski and Sacchi (1987). Poly (A)⁺ RNA was iso-

lated with biotinylated oligo(dT) and streptavidin paramagnetic particles, following the manufacturer's protocol (PolyA Tract System; Promega, Madison, WI). Total RNA (1 to 5 µg) was analyzed by Northern blot hybridization as described previously (Harrison and Dixon 1993).

High-molecular-weight DNA was extracted from *M. truncatula* leaves by the method of Dellaporta et al (1983). Southern blots were prepared by standard procedures (Sambrook et al. 1989) and hybridized as described in the Gene Screen Manual (NEN, Boston).

³²P-labeled probes were prepared from the cDNA inserts from Mt.AM1, Mt. AM2, Mt.AM3, MtPT1 (Liu et al. 1998), Gv1 (Burleigh and Harrison 1998), and pSR1-2B3 (18S rRNA; Eckenrode et al. 1985) by standard methods (Sambrook et al. 1989).

cDNA library construction.

Poly (A)⁺ was prepared from 8-week-old *M. truncatula* roots that were heavily colonized (70% of root length) with *G. versiforme* (Mt/Gv) and from noncolonized control roots (Mt). cDNA was synthesized from 5 µg of poly(A)⁺ RNA and cloned unidirectionally into the bacteriophage lambda Uni-Zap vector (Stratagene, La Jolla, CA). DNA was prepared from the control library (Mt) by the plate lysate method (Sambrook et al. 1989). The DNA was linearized with Eco01091, and RNA was transcribed in vitro (Megascript; Ambion) in the presence of UTP-biotin diluted 1:7.5 with UTP and a trace amount of ³²P-UTP (800 Ci/mmol) (0.625 µM final concentration). Phagemids containing single-stranded (ss) DNA were obtained from the Mt/Gv library by in vivo excision with 2×10^8 XL1 Blue cells coinfecting with 2×10^7 PFU lambda phage and 10^6 PFU ExAssist (Stratagene) helper phage (Schweinfest et al. 1990). ss circular DNA was isolated by standard procedures (Sambrook et al. 1989) and further purified by electroelution.

ssDNA (0.5 µg) from the Mt/Gv library, 13.5 µg of biotinylated Mt RNA, and 0.5 µg of poly(A) (19- to 24-mers) were lyophilized and redissolved in 2 µl of H₂O. In a control sample, biotinylated Mt RNA was replaced with an equal amount of biotinylated control RNA (pXef-1, Megascript kit; Ambion). Hybridization buffer (2× working strength) was added to give a final concentration of 0.5 M NaH_2PO_4 pH 7.2, 0.25% SDS (sodium dodecyl sulfate), and 1.25 mM EDTA. The nucleic acids were heated for 3 min at 100°C and incubated for 24 h at 68°C (under oil) to allow hybridization of complementary sequences. Streptavidin binding buffer (150 µl; 10 mM Tris HCl pH 7.4, 0.4 M NaCl, 2 mM EDTA and 0.3 mg of streptavidin per ml) was added and the samples were incubated for 30 min at 37°C. Hybridized sequences and remaining biotinylated RNA were removed by two phenol extractions and the nonhybridized, subtracted ssDNA was recovered by ethanol precipitation in the presence of 7.5 µg of carrier tRNA. Subsequently, a second round of subtractive hybridization was carried out as described above and the ssDNA was transformed directly into Epicurian Coli XL2-Blue ultracompetent cells (Stratagene) (transformation efficiency was 0.5×10^6 with unsubtracted ssDNA). Subtraction resulted in a 60% reduction in number of clones, compared with the control library. This corresponded well with the approximately 2.5-fold enrichment of an abundant fungal clone Gv1 (Burleigh and Harrison 1998) in the subtracted library, compared with the unsubtracted library.

Screening procedures.

Recombinants from the subtracted library were plated at low density (1,000 per 150-mm plate) and duplicate colony lifts were differentially screened with ^{32}P -labeled cDNA from control (Mt) and colonized roots (Mt/Gv). Colonies that showed increased signal with the Mt/Gv probes and colonies with no signal with either probe ("cold colonies"; Hodge et al. 1992) were selected and stored in microtiter plates. Five hundred seventy-six selected clones were grown, DNA was isolated, and duplicate dot blots were prepared and rescreened as described above.

DNA sequencing and analysis.

DNA sequencing was carried out by the dideoxy sequencing method with a *Taq* DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, LOCATION). The products were processed by an ABI373A automated DNA sequencer (Applied Biosystems). Sequences comparisons with the NR GenBank data base were performed by BLASTX searches. Protein sequence alignments were performed with PCGene (Intelligenetics) and GCG (Oxford Molecular) alignment programs. The nucleotide sequences reported will appear in the GenBank and EMBL data bases under the following accession numbers: Mt.AM1, AF106929; Mt.AM2, AF093506; Mt.AM3-1, AF106930; and Mt.AM3-2, AF106931.

PCRs.

Primers were designed to Mt.AM1 (5'-ctcgtgtgttgattgcctg, 5'-cttttag ctgcatctccatctggtg), Mt.AM2 (5'-tgccaacagagctagaa gaggc, 5'-gggttcgtatgaacctgtg), Mt.AM3 (5'-CAGGAAAG GTTGCAAAGAAGACG, 5'-CCACTCCCACCAGCTTGA) and Gv1 (5'ggtcacattaatcacttattgaa, 5'-ggtgctgtcaaggaaactatc). Control reactions contained 1 ng of the appropriate Bluescript plasmid containing the cDNA inserts. Genomic DNA reactions contained 50 ng of genomic DNA. The total volume of the reactions was 25 μl . Annealing of the Mt.AM1 primers was at 62°C, Mt.AM2 at 55°C, Mt.AM3 at 50°C, and Gv1 at 55°C. The reactions were amplified for 35 cycles. Samples (1 μl) of these amplification reactions were then re-amplified for an additional 35 cycles (total reaction volume, 25 μl). The products of the reactions were separated by gel electrophoresis, stained with ethidium bromide, and viewed by UV transillumination. The second round of amplification was only necessary to visualize the GV-1 product amplified from the *G. versiforme* genome by UV transillumination; however, all of the reactions were subjected to two rounds of amplification to ensure that the treatments were equal. The gels containing the products of the double amplifications were blotted and probed with the appropriate cDNA probes under standard conditions (Sambrook et al. 1989).

In situ hybridization.

Plant material was harvested, fixed, and embedded as previously described (Harrison and Dixon 1994). Mt.AM1 was digested with *Hind*III and *Eco*RI to produce a 775-bp internal fragment lacking a poly A tail. This insert was subcloned into pBluescript. DNA from the Mt.AM1 H/E subclone was linearized by digestion with either *Hind*III or *Eco*RI and used as a template for the in vitro synthesis of digoxigenin-labeled antisense and sense RNA probes. Probe synthesis and in situ hybridization of mycorrhizal and control root sections were as previously described (Harrison 1996).

In situ hybridization in whole root pieces.

This procedure was carried out as described by de Almeida Engler et al. (1994) with minor modifications. Roots were fixed in fixation buffer for 60 min, dehydrated as described, and stored in ethanol at -20°C. The prehybridization treatments were as described except for the proteinase K digestion. Proteinase K concentration was increased to 400 $\mu\text{g}/\text{ml}$ and digestion carried out for 60 min. Digoxigenin-labeled probes were prepared as described for regular in situ hybridization. Hybridization solution was 5 $\times$ SSPE (1 $\times$ SSPE is 0.15 M NaCl, 10 mM NaPO₄, and 1 mM EDTA [pH 7.4]), 50% formamide, 500 μg of tRNA per ml, and 50 μg of heparin per ml, and the probe at a final concentration of 300 ng/ml. Hybridization was at 52°C. Post-hybridization RNase A treatment was at a final concentration of 25 $\mu\text{g}/\text{ml}$ and the post-RNase treatment washes were in NTE (500 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA) containing 10 mM DTT (dithiothreitol). All experiments contained both antisense and sense samples treated in an identical manner.

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