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ORIGINAL PAPER

Medicago truncatula gene responses specific to arbuscular mycorrhiza interactions with different species and genera of Glomeromycota

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Abstract Plant genes exhibiting common responses to different arbuscular mycorrhizal (AM) fungi and not induced under other biological conditions have been sought for to identify specific markers for monitoring the AM symbiosis. A subset of 14 candidate *Medicago truncatula* genes was identified as being potentially mycorrhiza responsive in previous cDNA microarray analyses and exclusive to cDNA libraries derived from mycorrhizal root tissues. Transcriptional activity of the selected plant genes was compared during root interactions with seven AM fungi belonging to different species of *Glomus*, *Acaulospora*, *Gigaspora*, or *Scutellospora*, and under widely different biological conditions (mycorrhiza, phosphate fertilization, pathogenic/beneficial microbe interactions, incompatible plant genotype). Ten of the *M. truncatula* genes were commonly induced by all the tested AM fungal species, and all were activated by at least two fungi. Most of the plant genes were transcribed uniquely in mycorrhizal roots, and several were already active at the appressorium stage of fungal development. Novel data provide evidence that common recognition responses to phylogenetically different Glomeromycota exist in plants during events that are unique to mycorrhiza interactions. They indicate that plants should possess a mycorrhiza-specific genetic program which is comodulated by a broad spectrum of AM fungi.

Keywords *Medicago truncatula* · Glomeromycota · Transcript profiling · Mycorrhiza-specific gene expression · In situ hybridization

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Introduction

Plant roots form different types of beneficial interactions with soil microorganisms, the most common of which is the symbiotic arbuscular mycorrhiza (AM) association with fungi of the phylum Glomeromycota (Schüssler et al. 2001). The AM symbiosis probably appeared in primitive land plants around the Ordovician period (Redecker et al. 2000) and has persisted in newly evolved plant species to become nowadays an essential component of nutrient cycling and plant health in most terrestrial ecosystems (Smith and Read 1997). Despite the widespread nature of AMs and their importance for plant health and fitness

(Gianinazzi et al. 2002), information about molecular events or gene networks which are specific to this symbiosis is still fragmentary. Development and function of AM require the coordination of complex cellular processes between the eukaryotic partners. Two major, tissue-dependent steps are the elicitation at the root surface of an appressorium, required for fungal penetration of host roots (Giovannetti et al. 1994), and the differentiation within inner cortical cells of haustoria (arbuscules) which are assumed to be the major site of nutrient exchange between fungal and plant cells (Gianinazzi-Pearson 1996). Because AMs represent such a widespread and ancestral plant–microbe interaction, it has been proposed that some of the underlying mechanisms may have been recruited in the establishment of more recently evolved root compatibility with other symbiotic or parasitic microorganisms (Gianinazzi-Pearson 1997; Güimil et al. 2005; Kistner et al. 2005).

Legumes have the capacity to form root symbioses with both AM fungi and nodulating rhizobacteria, and they have therefore been a target for comparative profiling of plant gene expression in relation to AM formation and nodulation. While some overlaps have been reported in plant genes responding to the two microbial symbionts (Albrecht et al. 1999; Journet et al. 2002; Manthey et al. 2004; Sanchez et al. 2004, 2005; Weidmann et al. 2004; Hohnjec et al. 2005), these are not as frequent as may be expected considering the analogies in the root infection processes and the existence of common plant genes controlling both types of symbioses (Gianinazzi-Pearson 1997; Oldroyd and Downie 2004). On the other hand, conserved root responses to mycorrhizal and pathogenic fungi have been reported (Gianinazzi-Pearson et al. 1996; Liu et al. 2003), and comparative transcriptomics of rice have indicated that the symbiotic machinery recruited for AM may be shared up to 40% with pathogenic infections (Güimil et al. 2005). Likewise, the similar activation of signal transduction-related *Medicago truncatula* genes in response to an AM fungus and a growth-promoting *Pseudomonas fluorescens* points to common molecular pathways in the perception of the two microbes (Sanchez et al. 2005).

Transcriptome profiling based on molecular techniques such as macro/microarrays and suppressive subtractive hybridization has provided an extensive list of different plant genes that are predicted to be upregulated during AM interactions (Liu et al. 2003; Wulf et al. 2003; Brechenmacher et al. 2004; Gianinazzi-Pearson et al. 2004; Küster et al. 2004; Manthey et al. 2004; Weidmann et al. 2004; Güimil et al. 2005; Frenzel et al. 2005; Hohnjec et al. 2005). Previous expression analysis using Mt6k-RIT microarrays of cDNA probes from noninfected roots, young nodulated tissues, and roots colonized by *Glomus intraradices* gave 57 upregulated genes in *Glomus mosseae*-colonized *M. truncatula* roots, suggesting activation of common cell programs by these AM fungi (Gianinazzi-Pearson et al. 2004). Evidence for the existence of an even wider overlap has recently been provided by probing of 16k *M. truncatula* oligo microarrays which identified 201 plant genes as potentially coinduced in *G. intraradices* and *G. mosseae*-colonized root tissues, of which 29 have ESTs uniquely present in mycorrhizal root cDNA libraries (Hohnjec et al. 2005). Other searches for genes expressed in AM symbiosis by *in silico* screening of *M. truncatula* cDNA libraries (composed of ESTs from different tissues) have also revealed a number of plant ESTs only present in libraries derived from mycorrhizal roots colonized by *G. intraradices* (MtBC, MtGi, MtAmp) or *Glomus versiforme* (MtMHAMP) (Gianinazzi-Pearson et al. 2004; Frenzel et al. 2005).

In contrast, remarkably few plant genes have been demonstrated to exhibit mycorrhiza-specific expression in that transcripts are detected in mycorrhizal but not non-mycorrhizal roots and are not induced by either phosphate or microorganisms other than AM fungi. Brechenmacher et al. (2004) identified four mycorrhiza-induced genes in *M. truncatula*, which are not responsive to P and do not respond to *Sinorhizobium meliloti* (Sanchez et al. 2004), while Güimil et al. (2005) have reported 12 rice genes expressed exclusively in *G. intraradices*-colonized roots that show no transcriptional activity after treatment with P or after challenge with the fungal pathogens *Fusarium moniliforme* or *Helminthosporium grisea*. To pinpoint plant genes representing specific markers of the AM genetic program, we have focused on a subset of ESTs among mycorrhiza-responsive *M. truncatula* genes identified in Mt6k-RIT microarray analyses and exclusive to cDNA libraries derived from mycorrhizal roots (Gianinazzi-Pearson et al. 2004). Induction of expression has been evaluated for interactions with AM fungi belonging to different species or genera, and comparative transcriptomics have been performed under widely different biological conditions (phosphate fertilization, pathogenic or beneficial microbe interactions).

Materials and methods

Plant production and RNA isolation

Seeds of *M. truncatula* Gaertn. cv Jemalong, wild type (J5), and the mycorrhiza-defective genotype TRV25 (*Mtsym13/dmi3-1*; Catoira et al. 2000; Morandi et al. 2005) were surface-sterilized in 97% sulfuric acid (6 min), 96% ethanol (5 min), and 3% calcium hypochlorite (10 min) and rinsed in sterile distilled water. Germination was performed on 0.7% Bactoagar (Difco Laboratories, Detroit, MI, USA) at

25°C in the dark during 48 h. Seedlings were individually transplanted into 75 g of a sterilized (180°C, 6 h) mix of Terragreen® (OilDri-US special, Mettmann, Germany) and a neutral clay loam soil (2:1) for noninoculated plants, or into a mix (2:1) of Terragreen® and a soil-based inoculum (spores, hyphae, roots) of *G. intraradices* Schenck and Smith (BEG141), *G. mosseae* (Nicol. and Gerd.) Gerdemann and Trappe (BEG12), *G. geosporum* (Nicol. and Gerd.) Walker (BEG11), *G. versiforme* (Karsten) Berch (BEG47), *Gigaspora rosea* Nicolson and Schenck (BEG9), or *Scutellospora castanea* Walker (BEG1). For *Acaulospora longula* Spain and Schenck (BEG8), a mixture of Terragreen® (OilDri-US special, Mettmann, Germany) and sterilized "Marlins" soil (pH 5) (1:2) was used. Inoculum was produced on leek or clover and was previously checked for absence of *S. meliloti* by growing for 3 weeks with a hypernodulating *M. truncatula* genotype (TR122). Control seedlings were mock-inoculated with filtered washings (Whatman paper no. 3) of the mycorrhizal inoculum to reconstitute associated microflora. Plants were grown under constant conditions: 60–70% relative humidity, 16 h photoperiod, 19/22°C night/day, 320 µmol m^{-2} s^{-1} (LI-189 LI-COR radiation sensors, NE, USA), watered daily and twice a week with 5 ml Long Ashton nutrient solution (Hewitt 1966) without phosphate but containing double nitrate concentration to ensure no formation of nodules. Plants were harvested at 6, 21, or 35 days (depending on the AM fungus) after inoculation (days) for the J5 genotype and at 10 days for the TRV25 mutant. Fresh weights were recorded, and mycorrhiza development was assessed. Root systems of three randomly selected plants were digested in 10% KOH (90°C, 10 min) and stained 15 min in 0.05% trypan blue in lactoglycerol at 90°C. The number of appressoria on roots was estimated at 6 days for J5 and at 10 days for TRV25 after digesting in 10% KOH for 10 min and staining 5 min in 0.05% trypan blue in lactoglycerol. Intensity of cortex colonization and arbuscule frequency were estimated as described by Trouvelot et al. (1986) at 21 or 35 days for J5 using the MYCOCALC program (http://www.dijon.inra.fr/mychintec/Mycocalc-prg/download.html).

To assess whether improved phosphate nutrition could induce changes in plant gene expression, noninoculated wild-type *M. truncatula* plants were grown as described above and fertilized twice a week with 5 ml Long Ashton solution containing 5 mM Na_2HPO_4 during 21 days before harvest. To compare plant gene responses to beneficial bacteria, germinated seedlings of wild-type *M. truncatula* were inoculated with 10^6 CFU/ml of either *S. meliloti* strain RCR 2011 or *P. fluorescens* strain C7R12 in Long Ashton solution without nitrogen or enriched in iron, respectively (Sanchez et al. 2004). Control plants received the corresponding modified Long Ashton solution. Inoculated and noninoculated plants were harvested for each treatment at 21 days for the bacterial interactions.

A pathosystem was developed for studies on interactions between *M. truncatula* and the root pathogen *Fusarium acuminatum* Ellis and Everhart (strain DMS62148). Seeds were surface-sterilized and germinated for 48 h as described above. Seedlings were transplanted individually into 2 ml of sterile Long Ashton solution in a Multiwell™ tissue culture plate (Dominique Dutscher SA, France) and grown under gnotobiotic conditions (16 h photoperiod, 19/22°C night/day, 360 µmol m^{-2} s^{-1}). Roots of 10-day-old plants were inoculated with a spore suspension of *F. acuminatum* (10^5/ml) in water, plants were harvested at 2 or 4 days, and the fungal pathogen was detected by staining in 0.03% trypan blue.

RNA isolation and transcript profiling

Harvested tissues were stored in liquid nitrogen. For each treatment, total RNA was isolated from different pools of roots from three replicate experiments using the RNeasy® Plant Mini Kit (Qiagen, Hilden, Germany; 2, 4, and 6 days) or according to Franken and Gnädinger (1994; 21 days). Integrity of RNA was checked on agarose gels, concentration was determined from absorption values at A_{260} and A_{280}, and preparations were stored at −80°C until use. cDNA was prepared from 2 µg total RNA added to 1.5 µg oligodT, $dNTP_{15}$ (2.5 mM each) and made up to a final volume of 11.5 µl with sterile distilled water. RNA was denatured for 5 min at 70°C, placed on ice, and 5 µl MMLV (units moloney murine leukemia virus), 5× reaction buffer, 300 U MMLV RT, and 80 U RNase inhibitor were added. First-strand cDNA was synthesized at 25°C for 15 min followed by 50 min at 42°C and 2 min at 96°C.

Fourteen mycorrhiza-responsive *M. truncatula* genes were selected from Mt6k-RIT microarray analyses (Table 1) (Gianinazzi-Pearson et al. 2004), and the distribution of corresponding ESTs was determined in available *M. truncatula* cDNA libraries (http://medicago.toulouse.inra.fr/Mt/EST/) by *in silico* screening (Journet et al. 2002). Gene transcription was analyzed by RT-PCR as described by Brechenmacher et al. (2004). Gene-specific fragments were amplified by PCR using the primers, annealing temperature and number of cycles presented in Table 2. Amplification products were analyzed by 1.5% agarose gel electrophoresis stained with ethidium bromide and quantified in a Storm 860 phosphorimager with Image Quant software (Molecular Dynamics, Amersham Biosciences, Orsay, France). Expression levels of the different genes were normalized against the *Mtgap1* gene (encoding a glyceraldehyde phosphate dehydrogenase) of *M. truncatula* which has previously been shown to be an appropriate active reference control for equivalent reverse transcription

Table 1 Genes selected from Mt6k-RIT microarray analyses (Gianinazzi-Pearson et al. 2004) and present in mycorrhizal root cDNA libraries of *M. truncatula* (MtAMP, MtBC, MtHAM)

Gene ID (MENS)	Matching predicted protein	Gene function category	*M*-value (SE)
MtC10430	Nodulin 26-like	Membrane transport	1.29 (0.10)
MtC00638	Pi transporter	Membrane transport	1.27 (0.03)
MtC00512	Cytochrome P450	Secondary metabolism	1.24 (0.04)
MtC00285	Cysteine-rich antifungal protein	Defense and cell rescue	1.26 (0.19)
MtC00422.2	Kunitz-type trypsin inhibitor 1	Defense and cell rescue	1.31 (0.13)
MtC00162	Kunitz-type trypsin inhibitor 2	Defense and cell rescue	1.31 (0.18)
MtC91818	Kunitz-type trypsin inhibitor 3	Defense and cell rescue	1.23 (0.10)
MtC91337	Serine protease	Protein synthesis and processing	1.33 (0.26)
MtC91610	Subtilisin inhibitor	Protein synthesis and processing	1.16 (0.07)
MtC91267	Legume lectin	Miscellaneous	1.63 (0.38)
MtC91432	Lipase precursor	Miscellaneous	1.2 (0.02)
MtC91869	Mlo protein homolog	Unknown function	1.3 (0.14)
MtC10539	Unknown function	Unknown function	1.22 (0.08)
MtC00157	No homology	No homology	1.26 (0.16)

M-value is $\log^2$ of the expression ratio of mycorrhizal vs nonmycorrhizal roots (Dudoit et al. 2002).*MtAMP* cDNA library of *M. truncatula* root harvested 5 weeks postinoculation with *Glomus intraradices*; *MtBC* cDNA library of *M. truncatula* root harvested 3 weeks post inoculation with *Glomus intraradices*; *MtHAM* cDNA library of *M. truncatula* root harvested 10, 17, 22, 31, and 38 days postinoculation with *Glomus versiforme*; *MENS* cluster number according to the *Medicago* EST Navigation System (http://medicago.toulouse.inra.fr/Mt/EST)

Table 2 Oligonucleotide primer sequences, PCR conditions and number of cycles used for gene expression analyses by semiquantitative RT-PCR

Gene ID (MENS)	Primers 5′–3′ (forward and reverse)	PCR annealing temperature (°C)	Number of PCR cycles
Nodulin 26-like (MtC10430)	TAATAGCAGAAATGGTGGGC CGGCGACAGCAAATATATCC	55	30
Pi transporter (MtC00638)	GTGAGGTCAACATGCCATGC ACAAACATGGATGTTGCAGC	60	30
Cytochrome P450 (MtC00512)	TCTGGAAGAAGGATATGTGC GCGCAAGAGATTATACTAGC	57	30
Cysteine-rich antifungal protein (MtC00285)	TGCATCAATGTGGAACATGC TCTATCATGTGCAGAGATGC	57	30
Kunitz-type trypsin inhibitor 1 (MtC00422.2)	GTGAAATTCACTCCTTTCGC GCACGCCTCTGTAGGGCACC	60	30
Kunitz-type trypsin inhibitor 2 (MtC00162)	TTAGACCTGCTATCACAGGC ATCTCCAGTCTGTTGACTGC	60	30
Kunitz-type trypsin inhibitor 3 (MtC91818)	TGGCCCTCGATGGTGGTGCC CGAATAGAATACATGTCTCG	60	30
Serine protease (MtC91337)	GGGTTTGTTGGAAGAGTAG CAAGAATGTTCAATCCAGGC	55	30
Subtilisin inhibitor (MtC91610)	TCAGACCTGCTATCACCGGC TTCTGTTGACTGTCCACAGC	57	30
Legume lectin (MtC91267)	TGATTCAGTTTCAACTGCGG CAACTCTAACCCACTCAGGC	55	30
Lipase precursor (MtC91432)	ATGATGCATATTGGTTAGGC CCTAGAAGCATTTATGCAGC	57	30
Mlo protein homolog (MtC91869)	CCATTGGATCATCTTCTTGA AGGCTACCCCAAGGACAACC	60	30
Unknown function (MtC10539)	AGGAAAACAAGGGGACGGCT TTAGCGACACTTGACTTGGG	60	30
No homology (MtC00157)	ACCCTCACTTATTCTGCTGC GTACACAACAACTTAAAGGC	55	30
Mtgapdh1 (MtC00030)	TGAGGTTGGAGCTGATTACG AGCCTTGGCAGCTCCAGTGC	56	22

to cDNA and equivalent PCR amplification of mRNA from mycorrhiza tissues (Brechenmacher et al. 2004; Weidmann et al. 2004; Sanchez et al. 2005). Constitutive levels of *Mtgap1* transcripts were consistently checked on cDNA synthesized from RNA of *M. truncatula* roots from each biological condition. To confirm the identity of gene products, PCR fragments were extracted from agarose using the gel extraction kit (Qiagen, Hilden, Germany), cloned into the pGEM-T vector according to Promega (Promega France, Charbonnière-les-Bains, France), and sequenced using T7 and SP6 primers (MWG-Biotech, Edersberg, Germany). The plant origin of genes was confirmed by PCR amplification of *M. truncatula* genomic DNA extracted from leaves using primers deduced from EST sequences (Table 1) as described by Brechenmacher et al. (2004).

Where gene expression was weak or not detected after ethidium bromide staining, ^{32}P dCTP was included in the PCR reaction to increase detection sensitivity of transcripts (>200–400 fold). The PCR reaction was performed as described above, with the exception that 10 μCi of α32P-dCTP [3,000 Ci/mmol] (Amersham Biosciences) was included in each reaction. After PCR, amplification products were separated from the labeled nucleotide by chromatography on a ProbeQuant™ G50 minicolumn (Amersham Biosciences) as recommended by the manufacturer. The amplification products were separated by gel electrophoresis as described above, ethidium bromide-stained, and semidry-transferred to a nylon membrane (Fuji medical X-ray film; Fujifilm) soaked in 2×SSC. Autoradiograms were obtained by exposing membranes for 4 to 16 h in the presence of an intensifier screen (Amersham) at −80°C.

In situ hybridization

Roots of *M. truncatula* J5 plants colonized by *G. intraradices* were sampled at 21 days, and fragments were fixed overnight in glacial acetic acid/absolute ethanol (1:3, *v*:*v*), dehydrated through a graded ethanol series, and embedded in Paraplast plus (Sigma-Aldrich, St Quentin Fallavier, France). Semi-thick sections (10 μm) were mounted on RNase-free siliconed slides and deparaffinated and rehydrated for 5 min in 50 mM Tris–HCl (pH 7.6). The 600-bp clone MtBC15B02 in pBluescript SK-15B02 from the *M. truncatula*/*G. intraradices* MtBC cDNA library (Journet et al. 2002), corresponding to the EST MtC00285 (cysteine-rich antifungal protein), was digested with *Apa1* (Biolabs, France) and transcribed with T3-RNA-polymerase (sense probe) or digested with *Not1* (QBiogene, France) and transcribed with T7-RNA-polymerase (antisense probe). Sense or antisense RNA was digoxigenin(DIG)-labeled using the in vitro transcription protocol of Roche Diagnostics (Meylan, France). Root sections were hybridized in a humid chamber overnight at 46°C with 100 μg denatured sense or antisense probe in 0.15 M NaCl, 100 mM Tris–HCl (pH 7.6), 10% dextran sulfate (Pharmacia, France), 1× Denhardt's solution (Euromedex, France), and 50% formamide (Sigma-Aldrich). Sections were washed in 2× SSC and treated with RNase A (100 μg/ml) at 37°C for 30 min, then washed successively in 2× SSC, 1× SSC, and PBS containing 1% powered skimmed milk (Régilait, France) and 0.05% Tween 20 (Sigma-Aldrich). DIG-labeled RNA hybrids were detected immunologically using anti-DIG alkaline phosphatase-conjugated antibodies according to the supplier's protocol (Roche Diagnostics), and the enzyme revealed with nitro-tetrazolium blue and 5-bromo-4-chloro-3-indolyl phosphate. Bright-field photomicrographs (NOESIS, Les Ulis, France) of hybridized sections were taken with a tri-CCD DXC 390P Sony camera, which were observed in a DMRB microscope (Leica, Rueil Malmaison, France).

Results

Expression of *M. truncatula* genes at appressorium and arbuscule stages of interactions with *G. intraradices* or *G. mosseae*

At 6 days, *G. intraradices* and *G. mosseae* had developed an average of 3.5 and 2.8 appressoria per centimeter root, respectively, on root systems of the wild-type *M. truncatula* genotype J5. Three-week-old mycorrhizal plants, obtained using different batches of inoculum of *G. intraradices* or *G. mosseae*, gave consistently high levels of percentage root cortex colonization and arbuscule abundance (means presented in Table 3). Plant growth was enhanced by both fungi as compared to nonmycorrhizal mock-inoculated plants in the absence of phosphate fertilization (Table 3). No fungal structures were observed in association with mock-inoculated roots. Absence of nodules on root systems of inoculated plants was confirmed by microscope observation of the trypan-blue-stained root systems.

Of the 14 *M. truncatula* genes identified as being responsive to mycorrhiza development in Mt6k-RIT microarray analyses (Table 1), all except the nodulin 26-like gene were found to be exclusive to cDNA libraries derived from mycorrhizal roots. Consistent with this, only transcripts of the nodulin 26-like gene were significantly present in nonmycorrhizal mock-inoculated roots of *M. truncatula* (Fig. 1a,b). No transcripts of the remaining *M. truncatula* genes could be detected in noninoculated root extracts by RT-PCR after either ethidium bromide staining or ^{32}P labeling of amplification products (not shown), and they were detected uniquely in

Table 3 Total fresh weight and mycorrhizal development (means and SE) of *Medicago truncatula* J5, noninoculated or inoculated with different arbuscular mycorrhizal fungi

Treatment	Fresh weight/ plant (mg)	Intensity of root system colonization (*M*%)	Arbuscule abundance in the root system (*A*%)
Inoculated with:			
Glomeraceae			
Glomus intraradices[a]	534±30	68±9	40±5
Glomus mosseae[a]	601±42	66±5	38±7
Glomus geosporum[a]	721±43	69±4	44±4
Glomus versiforme[a]	792±35	63±7	42±6
Acaulosporaceae			
Acaulospora longula[b]	1767±262	66±8	43±7
Gigasporaceae			
Gigaspora rosea[a]	507±50	70±6	42±8
Scutellospora castanea[c]	3196±342	52±10	28±5
Noninoculated[a]+5 mM Na_2HPO_4	813±58	0	0
Noninoculated[a]	380±22	0	0
Noninoculated[b]	1493±281	0	0
Noninoculated[c]	2719±361	0	0

[a] Plants grown to 21 days
[b,c] Plants grown to 35 days

G. intraradices- or *G. mosseae*-inoculated roots (Fig. 1a,b). The plant genes encoding an inorganic phosphate transporter, cysteine-rich antifungal protein, subtilisin inhibitor, legume lectin, and two of unknown function (MtC00157, MtC10539) were already activated at 6 days in response to appressorium formation by both *G. intraradices* and *G. mosseae*. Appressoria of *G. intraradices* additionally induced serine protease and two Kunitz-type trypsin inhibitor (1, 2) gene transcription (Fig. 1a). Development of the AM fungi within root tissues further enhanced expression of the appressorium-activated *M. truncatula* genes and of the nodulin 26-like gene, and activated other genes, so that 11 of the *M. truncatula* genes were commonly expressed in roots colonized by *G. intraradices* or *G. mosseae* at 21 days. Three plant genes responded differently to the two AM fungi. Transcripts of the Mlo protein homolog and cytochrome P450 genes were only weakly detected using ^{32}P-PCR in *G. intraradices*- and *G. mosseae*-colonized roots, respectively, suggesting virtually no gene expression, and the Kunitz-type trypsin inhibitor 3 gene was not transcribed in presence of *G. mosseae* (Figs. 1b and 3).

Gene responses of *M. truncatula* roots to AM fungi from different genera

G. geosporum, *G. versiforme*, and *G. rosea* effectively colonized *M. truncatula* roots. At 21 days, they had reached levels of root colonization and arbuscule abundance similar to *G. intraradices* or *G. mosseae*, while mycorrhiza establishment took longer for *A. longula* and *S. castanea* (Table 3). Transcript analyses were therefore performed at 35 days for the latter two fungi. Ten of the 14 *M. truncatula* genes showed common expression profiles independent of the AM fungus colonizing roots and were activated in mycorrhizal roots formed by *G. geosporum*, *G. versiforme*, *G. rosea*, *A. longula*, or *S. castanea* (Fig. 2), as well as by *G. intraradices* and *G. mosseae*. The serine protease gene of *M. truncatula* responded to all the fungi but only very weakly to *S. castanea*, with a low ^{32}P-PCR signal (Fig. 3). Response of the cytochrome P450, Kunitz-type trypsin inhibitor 3, and Mlo protein homolog genes again differed depending on the species of AM fungus colonizing the *M. truncatula* roots (Figs. 2 and 3). Transcripts of the cytochrome P450 gene were not detected, even using ^{32}P-PCR, in roots colonized by *G. versiforme*, *G. rosea*, *A. longula*, or *S. castanea*. Likewise, the Kunitz-type trypsin inhibitor 3 gene did not respond to *G. versiforme* or *S. castanea*, and transcripts of the Mlo protein homolog gene were not detected in roots colonized by *G. geosporum* or *S. castanea* and only weakly, using ^{32}P-PCR, in presence of *A. longula* or *G. versiforme* (Fig. 3).

Influence of nonmycorrhizal conditions on AM-responsive *M. truncatula* genes

None of the 14 plant genes were activated by phosphate fertilization in noninoculated roots of *M. truncatula*. To determine whether a successful mycorrhiza interaction was necessary for the activation of the *M. truncatula* genes, transcript profiles were analyzed in roots of the mycorrhiza-defective genotype TRV25 which is mutated for the gene *DM13*/*MtSYM13*. In this mutant, AM development is blocked at the stage of appressorium formation at the root surface (Morandi et al. 2005). *G. intraradices* had developed approximately seven appressoria per centimeter of root in 10 days. At this time point, only the Kunitz-type trypsin inhibitor genes 1 and 2 were weakly activated in inoculated roots of the mutant (Fig. 4a). Nodulation of *M. truncatula* root systems by *S. meliloti* and rhizosphere colonization by *P. fluorescens* resulted only in the activation of the nodulin 26-like gene by both bacteria and of the Kunitz-type trypsin inhibitor 1 and serine protease genes by *S. meliloti* (Fig. 4b). Transcript accumulation could not be detected for any of the 14 *M. truncatula* genes in roots inoculated with *F. acuminatum* which became colonized by the fungal pathogen at 2 days and developed necrotic symptoms at 4 days. Absence of gene activation was confirmed using ^{32}P-PCR.

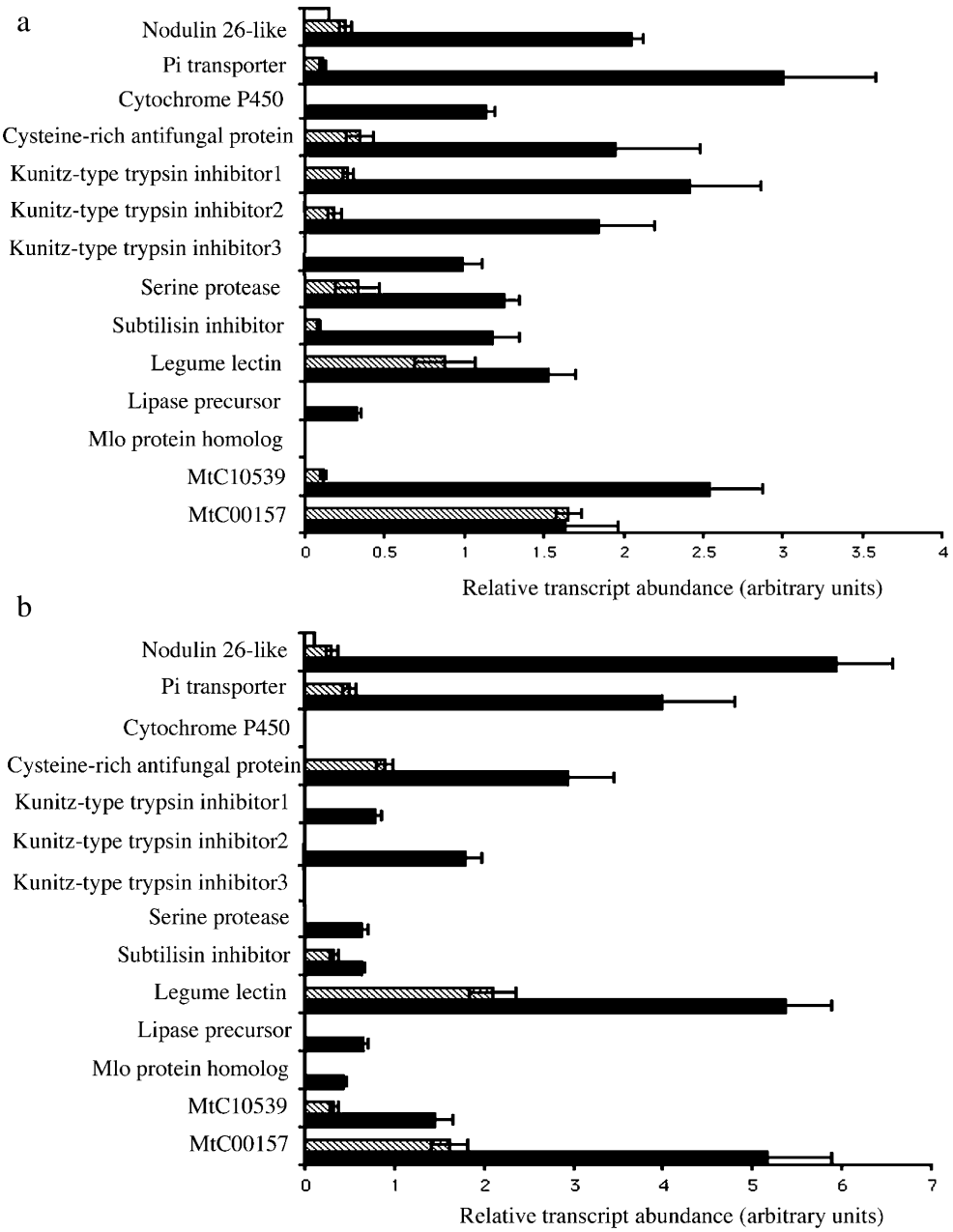

Fig. 1 Relative transcript abundance of 14 *Medicago truncatula* J5 genes in mock-inoculated (□) roots and in roots 6 (▨) or 21 (■) days after inoculation with **a** *Glomus intraradices* or **b** *Glomus mosseae*. *Bars* represent standard errors of means

Localization of cysteine-rich antifungal protein transcripts

To gain some insights into the implication of the consistent activation of an antifungal gene during AM interactions, two independent hybridization experiments were performed on sections of *G. intraradices*-colonized roots of *M. truncatula*. Arbuscules were visible as pale structures in control root sections hybridized with the sense probe for cysteine-rich antifungal protein transcripts (Fig. 5a,c). Hybridization of the antisense probe gave signals in cells containing arbuscules, indicating very localized gene activation, while cells without fungal structures showed no signals (Fig. 5b,d).

Discussion

There exist few reports of plant genes which are specifically expressed in mycorrhizal but not in nonmycorrhizal roots and that are not induced by either phosphate or microorganisms other than AM fungi. By focussing on a subset of *M. truncatula* ESTs present only in mycorrhiza cDNA libraries, we provide new data identifying ten mycorrhiza-induced plant genes which are expressed uniquely in mycorrhizal roots and that are not regulated by phosphate, beneficial bacteria, or a root pathogen, therefore representing mycorrhiza-specific candidate genes. None of the genes corresponds to the four *G. mosseae*-

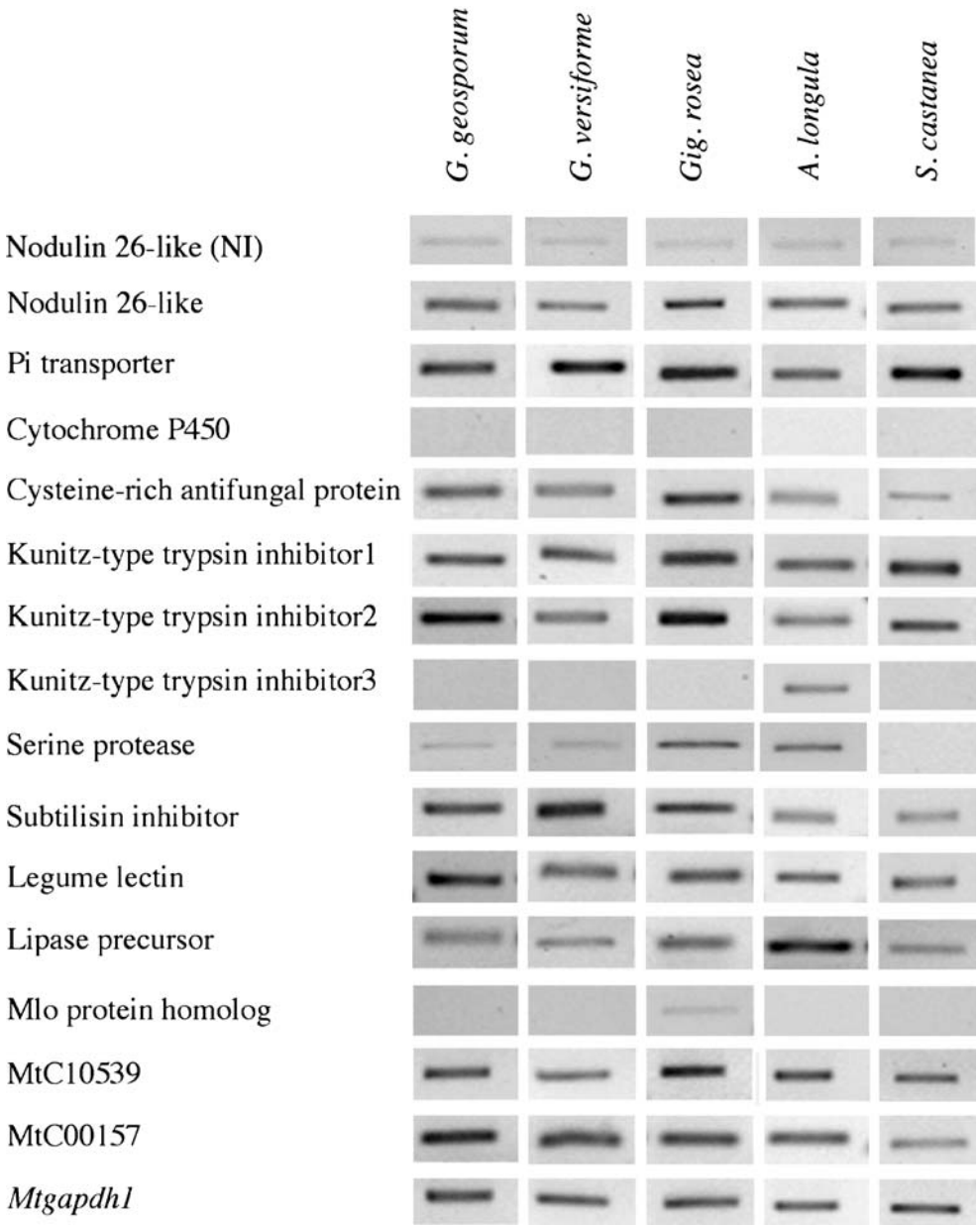

Fig. 2 Representative RT-PCR expression profiles for 14 *Medicago truncatula* J5 genes in mycorrhizal roots 21 days after inoculation with *Glomus geosporum*, *G. versiforme*, or *Gigaspora rosea* and 35 days after inoculation with *Acaulospora longula* or *Scutellospora castanea*. *Mtgapdh1* is used as a constitutively expressed gene. Expression of the nodulin 26-like gene in mock-inoculated plants (NI) is also shown

induced genes previously identified in *M. truncatula* and not responsive to P or *S. meliloti* (Brechenmacher et al. 2004; Sanchez et al. 2004). Neither are they homologs of the 12 rice genes reported by Güimil et al. (2005), which are expressed exclusively in *G. intraradices*-colonized roots and show no transcriptional activity after treatment with P or after challenge with the fungal pathogens *F. moniliforme* or *H. grisea*. Only the nodulin 26-like and the two Kunitz-type trypsin inhibitor genes showed transcriptional activity in interactions between *G. intraradices* and the incompatible *M. truncatula* genotype TRV25 mutated for the gene *DMI3/MtSYM13* (encoding a calcium and calmodulin-dependent protein kinase; Levy et al. 2004; Mitra et al. 2004), while nine genes were induced at a similar stage of the interaction of the wild-type genotype and *G. intraradices*. This observation could indicate that regulation of the Kunitz-type trypsin genes is located upstream of the *DMI3/MtSYM13* gene. The lack of induction of the nine other genes in the mycorrhiza-defective mutant further underlines the central role of the *DMI3/MtSYM13* gene in cell compatibility between mycorrhizal associates.

Although AM fungi are known to differ in their symbiotic capacities (Munkvold et al. 2004), few comparisons exist on the effect of different genera on host gene expression. Burleigh et al. (2002) described the effects of seven AM fungal species on root expression of four plant genes involved in host P-starvation responses, and

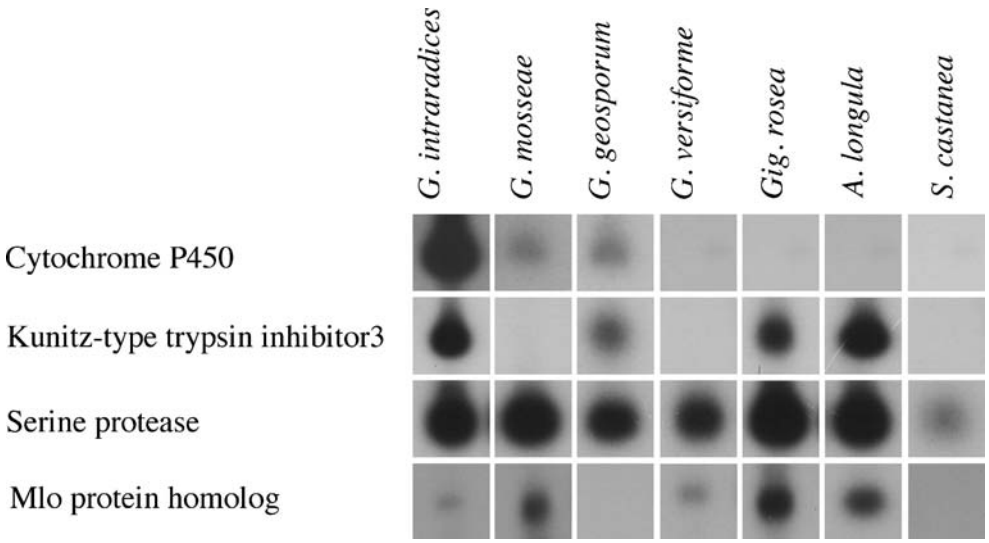

Fig. 3 ^{32}P RT-PCR expression profiles for four *Medicago truncatula* J5 genes in mycorrhizal roots 21 days after inoculation with *Glomus geosporum*, *G. versiforme*, or *Gigaspora rosea* and 35 days after inoculation with *Acaulospora longula* or *Scutellospora castanea*

Karandashov et al. (2004) described activation of the mycorrhiza-responsive *StPT3* phosphate transporter gene by several members of the Glomeromycota. In potato, the *StPT3* gene was not only expressed in arbuscule-containing cells but also in those harboring thick-coiled hyphae (Karandashov et al. 2004). These structures are typical of *Gigaspora* and *Scutellospora* species and could explain the relatively high expression of *MtPT4* in response to *Scutellospora* although the arbuscule abundance was lower. In the present study, we have identified 14 plant genes that are similarly induced in *M. truncatula* roots by two or more species of AM fungi. Most of these genes have considerable overlap in their expression profiles, being consistently activated by seven AM fungal species tested and by different genera. These results strengthen and extend the hypothesis of Hohnjec et al. (2005) that a basic genetic program must exist which is common to events that are essential for mycorrhiza interactions. At the same time, the variable responses of some plant genes to the different AM fungi point to the existence also of differential recognition

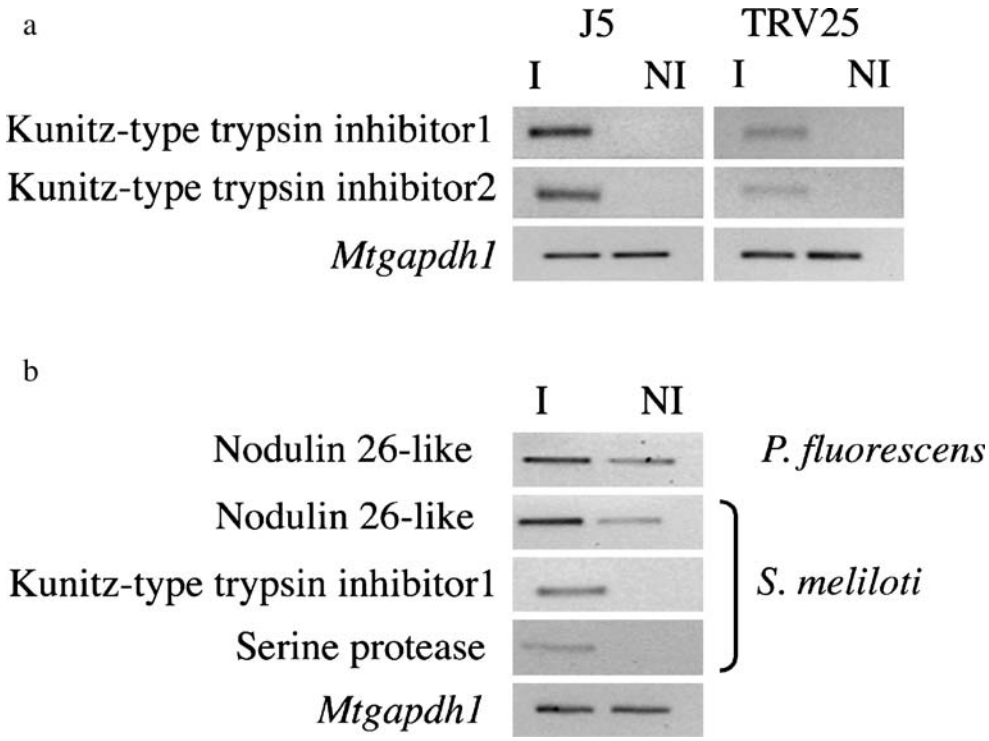

Fig. 4 RT-PCR analyses of *Medicago truncatula* gene expression in **a** *Glomus intraradices*-inoculated (*I*) and mock-inoculated (*NI*) roots of the mycorrhiza-defective genotype TRV25, 10 days after inoculation, and **b** in wild-type J5 roots, 21 days after inoculation (*I*) or not (*NI*) with *Pseudomonas fluorescens* or *Sinorhizobium meliloti*. *Mtgapdh1* is used as a constitutively expressed gene

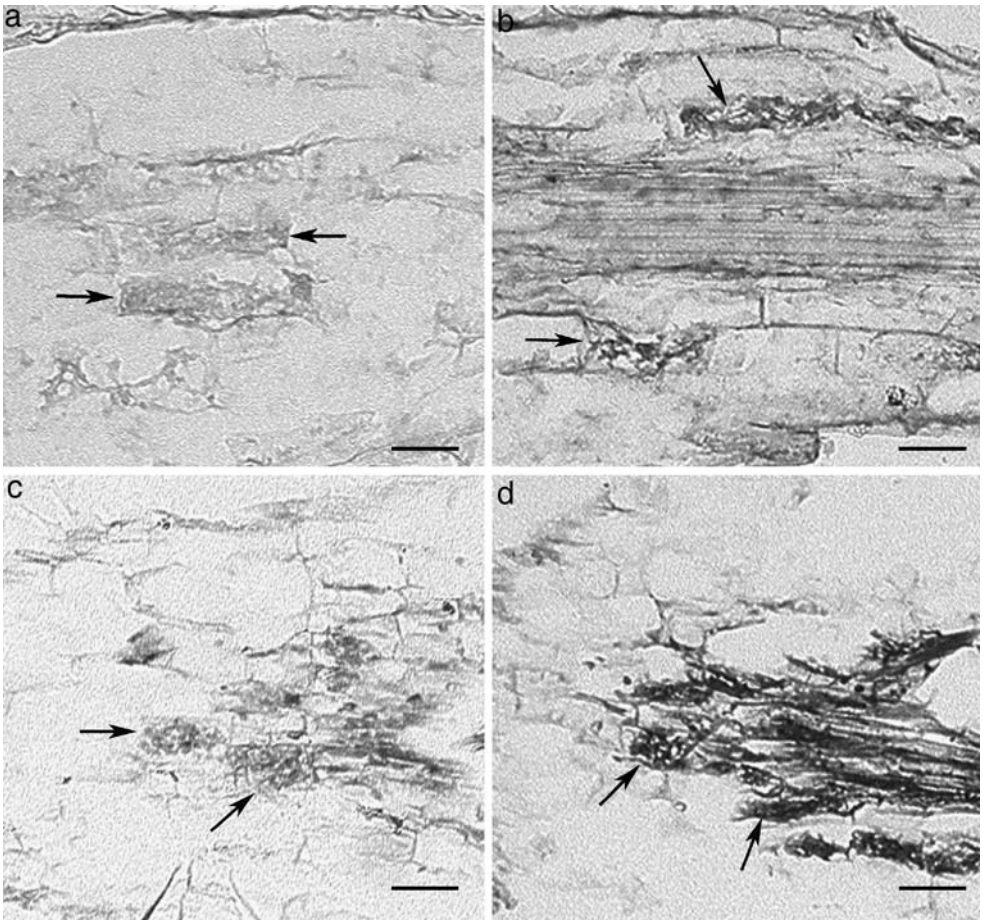

Fig. 5 Localization of transcripts of a cysteine-rich antifungal protein (defensin) gene in *Medicago truncatula* roots colonized by *Glomus intraradices*. Root sections were hybridized with digoxigenin-labelled sense (**a**,**c**) or antisense (**b**,**d**) probes. Arrows indicate cortical cells containing arbuscules. Bar=20μm

responses which, however, cannot be related to the fungal genus or species or to their effect on plant growth.

Only four of the *M. truncatula* genes that are consistently upregulated by the seven AM fungal species have been previously monitored in mycorrhizal interactions. Differential expression of the *M. truncatula* inorganic phosphate transporter gene *MtPT4* (MtC00638) has been identified in mycorrhizal roots colonized by *G. versiforme*, but not in nonmycorrhizal roots, and the encoded protein located in the periarbuscular host membrane (Harrison et al. 2002). The cysteine-rich antifungal protein gene (MtC00285) is the same class III defensin gene which has been reported to be induced in *M. truncatula* roots by *G. versiforme* and almost to the same extent as the phosphate transporter gene (Hanks et al. 2005), similar to that observed with the different AM fungi in the present study. Likewise, analyses of a novel family of lectin genes, including one corresponding to the EST MtC91267, in interactions with *G. intraradices* have indicated the existence of members which may play a role during arbuscule formation or function (Frenzel et al. 2005). Transcript analyses of the nodulin 26-like gene (MtC10430) also verify that its upregulation, although not AM specific, is a common phenomenon in roots of *M. truncatula* colonized not only by *G. intraradices* or *G. mosseae* (Brechenmacher et al. 2004; Manthey et al. 2004; Hohnjec et al. 2005) but also by AM fungi belonging to other *Glomus* species or Glomeromycota genera. The present observations confirm the mycorrhiza-related nature of this plant gene activation and show that it is part of a broad response to different AM fungi which may initiate very early during mycorrhizal interactions because all four genes are already active at the appressorium stage of root interactions with *G. intraradices* or *G. mosseae*.

A further six plant genes that are transcribed during the arbuscule phase of root colonization by the different AM fungi are also induced in response to *G. mosseae* or *G. intraradices* before intraradical mycelium develops. This is suggestive of downstream effects that are triggered by prepenetration events and which may have a role in modulating root interactions throughout the developmental phases of the fungi. Two of the appressorium-responsive genes encode proteins of unknown function, while others are serine protease, cysteine-rich antifungal protein (defensin), Kunitz-type trypsin inhibitor, and subtilisin inhibitor genes. Members of these gene families have been impli-

cated in plant defense to pests or pathogens. Serine proteases were among the proteins with endoproteolytic activity which increased in both mycorrhizal and *Aphanomyces*-infected roots of pea (Slezack et al. 1999). Both defensin and Kunitz-type trypsin inhibitors are antimetabolic proteins involved in defense strategies against predators (Garcia-Olmedo et al. 1998; Ussuf et al. 2001), and some plant subtilisin inhibitors are Kunitz-type trypsin inhibitors (Nielsen et al. 2004). Activation of serine protease, defensin, Kunitz-type trypsin inhibitor, and subtilisin inhibitor genes in mycorrhizal *M. truncatula* roots concords with already well-documented, defense-related gene expression in AM tissues (Gianinazzi-Pearson et al. 1996; Garcia-Garrido and Ocampo 2002). Certain defensins can have antifungal activity (Thomma et al. 2002; Wang and Ng 2006), and the implication of such a gene in the regulation of plant–fungal interactions in the AM symbiosis cannot be excluded. Members of the defensin gene family are differentially expressed in response to pathogens and during plant development, and, consistent with AM fungal appressorium-induced activation, gene products have been localized primarily in the outermost cell layers of plant tissues (Terras et al. 1995). However, in situ hybridization clearly indicates that defensin gene expression is subsequently associated with arbuscule development within inner root cortical cells. This adds to consistent observations that arbuscule-containing cells are sites of accumulation of plant defense gene products and raises the perennial question of the meaning of such procesnses for symbiotic interactions (Gianinazzi-Pearson 1996; Cordier et al. 1998). Different hypotheses include their implication in host control over intracellular fungal development, a role in the developmental reprogramming of colonized cell contents, or nonspecific symptoms of a general stress response to cell colonization (Garcia-Garrido and Ocampo 2002). The paradox remains because overexpression of defense genes, including defensins, in plants increases resistance to fungal pathogens but does not affect establishment of AM fungi within root tissues (Gianinazzi-Pearson et al. 1996; Turrini et al. 2004).

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