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Expression profiling of up-regulated plant and fungal genes in early and late stages of *Medicago truncatula-Glomus mosseae* interactions

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Abstract Suppression subtractive hybridization (SSH), expression profiling and EST sequencing identified 12 plant genes and six fungal genes that are expressed in the arbuscular mycorrhizal symbiosis between *Medicago truncatula* and *Glomus mosseae*. All the plant genes and three of the fungal genes were up-regulated in symbiotic tissues. Expression of 15 of the genes is described for the first time in mycorrhizal roots and two are novel sequences. Six *M. truncatula* genes were also activated during appressorium formation at the root surface, suggesting a role in this early stage of mycorrhiza establishment, whilst the other six plant genes were only induced in the late stages of mycorrhization and could be involved in the development or functioning of the symbiosis. Phosphate fertilization had no significant influence on expression of any of the plant genes. Expression profiling of *G. mosseae* genes indicated that two of them may be associated with appressorium development on roots and one with arbuscule formation or function. The other three fungal genes were expressed throughout the life-cycle of *G. mosseae*.

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Introduction

The ability to interact with arbuscular mycorrhizal (AM) fungi was a crucial step in the evolution of vascular land plants. Fossil data indicate that, at the time of land colonization, plants already harboured fungal endophytes forming intracellular branched structures similar to arbuscules (Redecker et al. 2000; Remy et al. 1994). It is, therefore, realistic to assume that AM formation was one of the early developmental programmes that evolved in plants. The programme must have recruited genes from already existing plant functions and modified their expression to fulfil the needs of AM formation and the symbiosis. This may have resulted in mycorrhiza-regulated members of gene families, as is typical of higher plants (Flavell 1980), or even in new genes with mycorrhiza-specific functions. As the analysis of mutants suggests, the mycorrhiza cell programme was probably the basis for the later evolution of the root symbiosis formed between legumes and the nitrogen-fixing rhizobia (Gianinazzi-Pearson and Denarié 1997). The ability of plants to form mycorrhiza has been lost during evolution in some cases, leading to the phenomenon of non-mycorrhizal plants, mainly in the Brassicaceae and Chenopodiaceae (Newman and Reddell 1987). Plant gene expression patterns during AM formation and functioning have been analysed over recent years in order to elucidate the developmental programme involved. Much of the research has been targeted at plant genes known to be active in interactions with pathogenic organisms (Blee and Anderson 2000; Gianinazzi-Pearson et al. 1996), with symbiotic rhizobia (Albrecht et al. 1999), or in processes involved in the uptake and transport of nutrients (Gianinazzi-Pearson et al. 2000; Harrison 1999a; Harrison et al. 2002; Rausch et al. 2001). In addition, non-targeted approaches have identified known genes not necessarily

expected to be involved in mycorrhization, as well as a few novel plant genes (Lapopin and Franken 2001).

The situation for the fungal partner is different. Analysis of rRNA sequences have shown that AM fungi form a monophyletic group dating back to a time coinciding with fossils of the first land plants (Remy et al. 1994; Simon et al. 1993). Because nothing is known about the biology of possible non-symbiotic progenitors of these fungi and because spore germination is the only developmental phase independent of the plant, all AM fungal genes have had to be newly identified. Targeted approaches have been directed towards cell wall synthesis, nutrient metabolism or transport across membranes, and non-targeted approaches have focussed mainly on plant-free tissues like germinating spores or extraradical hyphae (Franken and Requena 2000; Harrier 2001; Harrison 1999b; Requena et al. 2002). Only a few fungal genes have been identified from symbiotic tissue (Burleigh and Harrison 1998; Delp et al. 2000), probably because the frequency of fungal transcripts among the total symbiotic RNA population is very low (Maldonado-Mendoza et al. 2002). Thus, little is known about genes of AM fungi or about their expression patterns during development within host roots.

The rather limited information gained so far about plant and fungal gene expression during AM formation and function prompted us to exploit suppression subtractive hybridization (SSH) as an approach to identify novel plant and fungal genes active in an established symbiosis between *Medicago truncatula* and *Glomus mosseae*. SSH is a powerful technique that enables cloning of ESTs of regulated genes in two different RNA populations (Diatchenko et al. 1996). Being based on PCR, it increases the possibility of identifying fungal genes or weakly expressed plant genes in the symbiotic transcriptome. ESTs of up-regulated genes were cloned and sequenced to investigate how they could be involved in AM symbiosis development and/or functioning in the fully established symbiosis, in comparison to early steps of plant-fungal interactions in wild-type *M. truncatula*. Expression profiles were also analysed in phosphate fertilized plants in the case of plant genes, or in extraradical hyphae and germinating spores for fungal genes.

Materials and methods

Growth and inoculation of plants

Seeds of *Medicago truncatula* Gaertn. cv Jemalong line J5 were surface sterilized in sulphuric acid (6 min), 96% ethanol (5 min) and 3% calcium hypochlorite (10 min), then rinsed in sterile water. After germination for 48 h at 25°C in the dark on 0.7% Bacto-agar, seedlings were transplanted into a sterilized (twice for 2 h at 180°C) Terragreen:neutral soil mix (2:1) (Terragreen OilDri) for control plants and into Terragreen mixed (2:1) with a soil-based inoculum (spores, hyphae, mycorrhizal roots) of *Glomus mosseae* (Nicol. and Gerd.) Gerdemann and Trappe (isolate BEG 12), previously produced on leek plants, for mycorrhizal plants. The inoculum was tested for the absence of *Sinorhizobium meliloti* prior to use by growing with a *G. mosseae*-inoculated hypernodulating *M. truncatula* line (TR122) for 3 weeks. Non-inoculated seedlings were watered with a Whatman paper (no.°1)-filtered water suspension of the mycorrhizal inoculum. Plants were grown in a constant environment (16 h photoperiod, 320 μE m^{-2} s^{-1} irradiance, 19/22°C night/day, 60–70% RH), watered daily and twice a week with Long Ashton nutrient solution (Hewitt 1966) without phosphate but containing double nitrate concentration to ensure no formation of nodules. Plants were harvested at 5 and 20 days after inoculation (dai). To assess whether phosphate fertilization influenced plant gene expression, non-inoculated plants were grown for 3 weeks and fertilized twice a week with Long Ashton solution containing 5 mM Na_2HPO_4.

Aliquots of roots were stained with trypan blue to estimate mycorrhiza root colonization according to the method of Trouvelot et al. (1986) (http://www.dijon.inra.fr/bbceipm/Mychintec/New-Files/New.html). Remaining roots and leaves were stored in liquid nitrogen after harvest. Spores and external hyphae of *G. mosseae* were isolated as described by Butehorn et al. (1999).

RNA and DNA preparation

RNA was extracted from non-symbiotic and *G. mosseae*-inoculated *M. truncatula* roots using the method of Franken and Gnädinger (1994), and from external hyphae and spores of *G. mosseae* using the protocol described by Butehorn et al. (1999). Samples were treated with DNase by incubating at 37°C for 30 min 25 μg total RNA, 40 U RNase inhibitor, 25 U RNase-free DNase (Promega), 6 μl 10× buffer provided by the supplier of the enzyme and DEPC water to a final volume of 60 μl. The DNase was removed by phenol/chloroform/isoamyl alcohol (25:24:1) extraction and RNA was precipitated overnight at –20°C in 0.1 vol sodium acetate (3 M) and 2.5 vol ethanol (95%) and resuspended in DEPC water. mRNA was purified from total RNA using the Dynabeads mRNA Direct kit (Dynal).

DNA was extracted from *G. mosseae* spores after treatment in lysozyme (1 mg/ml of spore suspension in water) at 37°C for 1 h and washed in Tris-EDTA (TE) buffer. Spores were gently crushed in lysis buffer containing 50 mM Tris HCl pH 8, 25 mM EDTA, 50 mM NaCl, 1% SDS, 1% sarcosyl, 0.1% Triton X-100 and 10 mM β-mercaptoethanol. Proteins were removed by 50 μg/ml proteinase K (Sigma) treatment for 1 h at 60°C and nucleic acids were purified once with phenol/chloroform (1:1) and once with chloroform/isoamyl alcohol (24:1). DNA was precipitated at –80°C for 1 h in 0.1 vol 5 M ammonium acetate and 1 vol of pure isopropanol, centrifuged (10,000 *g*), washed with 70% ethanol and resuspended in TE buffer.

DNA was extracted from *M. truncatula* leaves by grinding in lysis buffer (100 mM Tris HCl pH 8, 50 mM EDTA, 500 mM NaCl, 1%SDS, 10 mM β-mercaptoethanol) and heating 10 min at 65°C. Proteins were precipitated with 0.3 vol 5 M potassium acetate for 20 min on ice and centrifuged 20 min at 2,500 *g* at 4°C. Nucleic acids were precipitated with 1 vol pure isopropanol and the pellet was resuspended in TE buffer. RNA was removed by RNase (Sigma) treatment (1/100 vol.) 30 min at 37°C. DNA was purified twice in phenol chloroform (1:1), once in chloroform and finally precipitated with 0.1 vol 3 M sodium acetate and 1 vol of isopropanol. After centrifugation, the pellet was washed with 75% ethanol and resuspended in TE buffer.

SSH library construction

The library was constructed using the PCR Select system according to the protocol provided by the supplier (Clontech). Briefly, 2 μg of mRNA from 20-day-old *G. mosseae*-colonized (Table 1; experiment 1) and non-mycorrhizal roots were used as template to synthesize two cDNA populations that were then digested with the *Rsa*I restriction enzyme. Two different adaptors carrying PCR and cloning sites were ligated to the mycorrhiza cDNA in order to obtain two populations of tester cDNA. No adaptors were added to

Table 1 Parameters of mycorrhizal colonization: proportion of cortex colonized by *Glomus mosseae* (M%) and relative arbuscule frequency (A%) in *Medicago truncatula* root systems. Plants were pooled in experiment 1

Experiment	M	A
1	46.8	37.3
2	61.4±10.3	57±9.8
3	48.5±21.1	34.2±15.4
4	76.3±8.4	47.6±7.2

non-mycorrhiza root cDNA (driver cDNA). After verification of the ligation efficiency, each denatured tester cDNA was subtracted by hybridizing it to half of the denatured driver cDNA followed by a second hybridization of the two subtracted tester cDNA samples. Thereafter, cDNA fragments corresponding to differentially expressed transcripts from the mycorrhiza cDNA were amplified and cloned into the pGEM-T vector (Promega).

Polymerase chain reactions

PCR was carried out in a 20-μl final volume containing 2.5 mM $MgCl_2$, 125 μM dNTP, 0.5 μM each primer, 0.5 U *Taq* polymerase (Gibco BRL) and 1× buffer provided by the supplier of the enzyme. Inserts from the SSH library clones were amplified from 1 μl of the 1:100 diluted boiled bacterial stock solution using M13for and M13rev primers in 30 PCR cycles for 1 min per temperature step (denaturing: 94°C, annealing: 58°C, elongation 72°C) and a final 5-min extension step at 72°C. To determine plant or fungal origin of ESTs, 1 ng of *G. mosseae* and of *M. truncatula* genomic DNA served as template in PCR reactions using primers deduced from EST sequences. After denaturation for 5 min at 95°C, 30 cycles were carried out with 45 s at 94°C, 45 s at the appropriate annealing temperature and 1 min at 72°C, followed by an additional 5 min at 72°C.

For semi-quantitative reverse transcription (RT) PCR, cDNA was synthesized in a final volume of 25 μl from 1 μg total RNA of inoculated and non-inoculated roots after DNase treatment using M-MLV reverse transcriptase (Promega). RNA and 1 μg oligodT_{15} were added to sterilized water to a final volume of 13 μl. After denaturing at 70°C for 5 min, the mixture was placed on ice and dNTPs (final concentration of 500 μM dATP, dGTP, dTTP and dCTP), 5 μl 5× M-MLV reaction buffer, 300 U M-MLV RT and 80 U RNase inhibitor were added (Promega). First strand cDNA was synthesized at 25°C for 15 min, followed by 1 h at 42°C, and the enzyme then inactivated 2 min at 95°C. Semi-quantitative RT-PCR experiments were carried out using 1 μl of a diluted 1/4 cDNA synthesis (1% of the RT reaction) and primers deduced from the EST sequences in 20, 23, 26, 29, 32, 35 and 38 PCR cycles with 45 s per temperature step (denaturing: 94°C, annealing: depending of the primers, elongation: 72°C) and a final 5 min extension step at 72°C. Primers of the *gapdh* gene were designed from the consensus sequence of the *Mtgap*1 cluster MtC00030_GC (http://medicago.toulouse.inra.fr/Mt/EST/DOC/MtB.html). Aliquots (7 μl) of PCR products were analysed by electrophoresis on 2% agarose gels, stained by ethidium bromide, and quantified using an ImageQuant software (Molecular Dynamics, Amersham Bioscience). For each biological condition, the same RT reaction was used to study expression profiling of all genes and three repetitions were performed on different root samples for quantification of PCR products.

Reverse Northern hybridization of cDNA arrays

^{32}P-labelled cDNA was synthesized from 2.5 μg total RNA of mycorrhizal and non-mycorrhizal roots as described above modified by the addition of 50 μCi α^{32}P dCTP and a concentration adjustment of the non-labelled dCTP to 5 μM. ^{32}P-labelled SMART cDNA probes were obtained from total RNA of spores and external hyphae of *G. mosseae* by reverse transcription as described in the SMART cDNA synthesis kit (Clontech). Aliquots (2 μl) first strand cDNA were labelled by 25 PCR cycles using the programme and mix composition provided by the supplier (Clontech). All probes were purified on ProbeQuant G-50 Micro Columns (Amersham Bioscience), denatured 5 min at 95°C and placed on ice.

PCR products of SSH clones were separated in 1.4% agarose gels, treated twice for 15 min with denaturing solution (0.5 M NaOH, 1.5 M NaCl), twice for 15 min with neutralizing solution (0.5 M Tris HCl pH 7.5, 1.5 M NaCl) and once for 20 min with 20× SSC (3 M NaCl, 300 mM sodium citrate pH 7), and then transferred to Hybond XL (Amersham Bioscience) and fixed by UV (70,000 μJ/cm^2). Membranes were prehybridized at 60°C for 1 h in Gibbert and Church hybridization buffer (Sambrook et al. 1989), hybridized overnight at 60°C, then washed twice in 2 SSC/0.1%SDS and in 0.5 SSC/0.1% SDS at 60°C, and twice in 0.5 SSC/0.1% SDS at 65°C. Hybridization signals were quantified using a Storm 860 Phosphorimager and ImageQuant software (Molecular Dynamics, Amersham Bioscience).

Northern blot analysis

Probes were labelled by PCR using 100 pg purified plasmid containing the insert of interest in a 20-μl final volume of 2.5 mM $MgCl_2$, 4.5 μM dATP, dTTP and dGTP, 50 μCi α ^{32}P dCTP, 0.5 μM of M13for and M13rev primers, 0.5 U *Taq* DNA polymerase (Invitrogen) and 1× buffer provided by the supplier. An initial denaturation at 95°C for 5 min was followed by 30 cycles at 94°C for 30 s, 58°C for 1.25 min, 72°C for 2 min, and a final extension step of 10 min at 72°C. All probes were purified as described above. An aliquot of 10 μg total RNA was denatured at 55°C for 15 min in buffer containing deionized formamide, formaldehyde, 10× MOPS electrophoresis buffer and ethidium bromide. RNA was separated in agarose gel containing formaldehyde at 50 V for 2 h in 1× MOPS electrophoresis buffer. rRNA was localized under UV light. Gels were rinsed 15 min in ultra-pure water, twice in 20 SSC for 15 min, and RNA transferred overnight by capillarity onto Hybond XL and fixed by UV. Membranes were prehybridized at 60°C for 1 h in Gibbert and Church buffer, hybridized overnight at 65°C, then washed at 65°C twice in 2SSC/0.1%SDS, 0.5SSC/0.1%SDS, and 0.25SSC/0.1%SDS.

DNA sequencing and analysis

ESTs were sequenced using T7 and SP6 primers (Genome Express Company, France). Sequences were deposited in the EMBL database and compared using the blastx (Gish and States 1993) algorithm with sequences in EMBL and the SwissProt databases. In order to identify longer sequences, searches were made in MtC and TIGR databases for homologies with near or full-length cDNAs obtained by clustering (http://medicago.toulouse.inra.fr/Mt/EST/DOC/MtB.html; http://www.tigr.org/tdb/tgi/mtgi).

Statistical analyses

Data from semi-quantitative PCR evaluations of gene expression were statistically compared between non-inoculated and *G. mosseae*-inoculated treatments (NI versus I), or between plants with or without phosphate fertilization (+P versus –P), using the Student *t* test.

Results

Mycorrhiza development

No arbuscules were present in root systems of *M. truncatula* J5 5 dai with *G. mosseae*, which developed an average of 1.5 appressoria per cm root. Three-week-old mycorrhizal plants obtained using different batches of inoculum of *G. mosseae* had colonization levels of 46.8–76.3%, and arbuscules were present in the roots (Table 1). Absence of nodules or nodule initials in *G. mosseae*-inoculated plants was confirmed by microscope observations of the trypan blue-stained root systems.

Characterization of the subtractive cDNA library

In order to establish an AM EST library enriched in fungal genes and in symbiosis-induced plant genes, cDNA from well-established *M. truncatula-G. mosseae* mycorrhiza (Table 1; experiment 1) was subtracted by cDNA from corresponding control roots. The presence of *Mtgap*1 cDNA sequences in subtracted and non-subtracted cDNA populations was analysed to confirm subtraction efficiency. This gene, which encodes a glyceraldehyde phosphate dehydrogenase, is constitutively expressed in non-symbiotic *M. truncatula* roots, and semi-quantitative RT PCR showed that its expression was unaltered in mycorrhizal roots (Fig. 1). Thirty PCR cycles using *Mtgap*1 primers gave only an amplification product of the expected size with non-subtracted cDNA of mycorrhizal *M. truncatula* roots, showing that the subtraction was efficient (data not shown).

An MtGmLs (*M. truncatula-G. mosseae* late-stage interactions) SSH library of 641 ESTs was obtained after amplification and cloning the subtracted cDNA from mycorrhizal roots. Initial screening of the cDNAs to remove false positives was performed by reverse Northern hybridization with cDNA probes of non-mycorrhizal and *G. mosseae*-colonized *M. truncatula* roots (Fig. 2) from a batch of plants different to those used to set up the SSH library (Table 1; experiment 2). Hybridization signal intensities were normalized using the *Mtgap*1 gene. ESTs corresponding to genes with at least threefold increases in transcript accumulation in mycorrhiza compared with control roots (Fig. 2) were sequence analysed. Eighteen different genes were identified after annotation and redundancy analyses (Table 2). A hybridization signal was obtained with cDNA from control roots for eight of the genes and no amplification was obtained by PCR on genomic DNA of *G. mosseae*, showing that these genes were of plant origin. The remaining 10 ESTs only gave a hybridization signal with cDNA from mycorrhizal roots. The fungal origin of six ESTs of these 10 was demonstrated by PCR of genomic DNA of *G. mosseae* and *M. truncatula* with primers defined for these ESTs (Fig. 3; Table 3). The remaining four ESTs corresponded to plant genes, which was also confirmed by PCR on plant and fungal genomic DNA (data not shown). Blastx searches

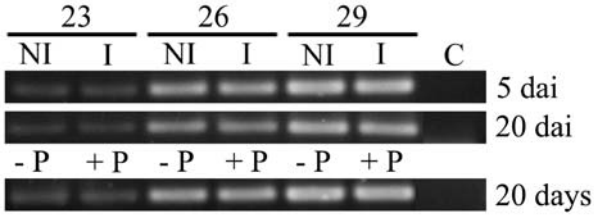

Fig. 1 Comparison of *gapdh* expression in roots of *Medicago truncatula* 5 and 20 days after inoculation (dai) (*I*) or not (*NI*) with *Glomus mosseae*, or 20 days with (+*P*) or without (–*P*) phosphate fertilization. Analysis by semi-quantitative RT-PCR of *gapdh* transcript levels using 1/4-diluted cDNA and 23, 26 and 29 amplification cycles. The amplified *gapdh* fragment corresponds to the expected size (144 bp)

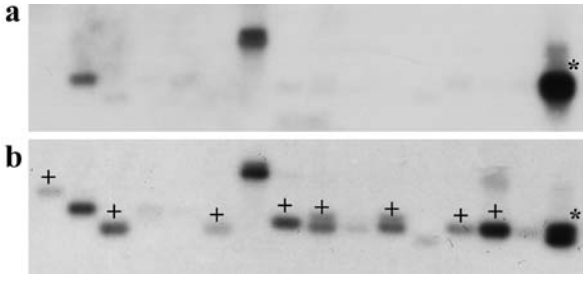

Fig. 2a, b Identification of ESTs corresponding to up-regulated genes. ESTs from the SSH library were transferred onto membranes and hybridized with ^{32}P-labelled cDNA probes from (**a**) non-mycorrhizal and (**b**) mycorrhizal root RNA. ESTs presenting a threefold increase in hybridization signal intensity with mycorrhizal probes are indicated by (+). Hybridization signals were normalized for quantification based on the *gapdh* signals (*) on autoradiograms where *gapdh* signals were not saturated

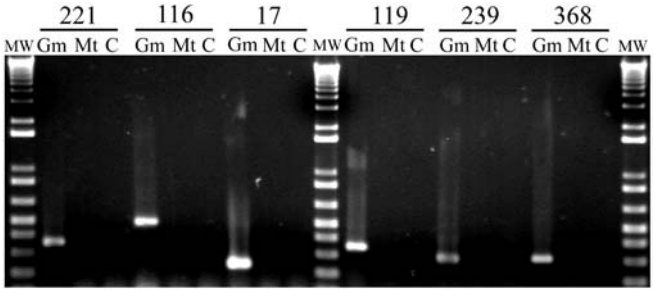

Fig. 3 Demonstration of the fungal origin of six ESTs by PCR on genomic DNA from *G. mosseae* (*Gm*) or *M. truncatula* (*Mt*) (*MW* molecular weight; *C* water control)

give sequence similarities for six of the 12 plant ESTs and two of the six fungal ESTs (Table 2). Putative functions were confirmed and sequence similarities were found for another eight ESTs by blastn analysis of annotated clusters (Table 2).

Table 2 Genes up-regulated at least threefold in *Medicago truncatula-Glomus mosseae* mycorrhiza as assessed by reverse Northern analysis

SSH ESTs	Accession number	Matching sequence (blastx) and E value	Origin of matching sequence	Clustering in the MtC and in the TIGR *M. truncatula* gene index (TC), blastn E value and annotation
Plant genes				
MtGmLs349	AJ311220	Glutamine synthetase ($2e^{-18}$)	*Medicago truncatula*	MtC00744 ($1.5e^{-82}$) glutamine synthetase, TC59781($1.8\ e^{-82}$) glutamine synthetase
MtGmLs494	AJ311232	Nodulin 26-like, multifunctional aquaporin ($9e^{-27}$)	*Medicago truncatula*	MtC10430 ($4.1e^{-70}$) nodulin 26-like, TC68574 ($7.8e^{-71}$) multifunctional aquaporin
MtGmLs5	AJ311228	PR 10 ($1e^{-3}$)	*Medicago sativa*	MtC10531 ($9.5e^{-58}$) probable PR10, TC71376 ($1.1e^{-57}$) weakly similar to PR10
MtGmLs31	AJ311234	Germin-like protein ($2e^{-10}$)	*Medicago truncatula*	MtC30458 ($2e^{-32}$) probable germin-like protein, TC60195 ($1.9e^{-64}$)
MtGmLs154	AJ311230	Probable wound induced protein ($3e^{-27}$)	*Arabidopsis thaliana*	MtC61255 ($1.9e^{-67}$), TC69789 ($4.4e^{-101}$) probable wound induced protein
MtGmLs618	AJ311225	Glutathione-S-transferase ($5e^{-43}$)	*Medicago truncatula*	MtC40051 ($4.2e^{-134}$) probable glutathione-S-transferase, TC59875 ($4.8e^{-134}$) glutathione-S-transferase like
MtGmLs11	AJ311239	No hits	–	MtC30403 ($1.3e^{-132}$), TC70845 ($5.1e^{-130}$)
MtGmLs164	AJ311247	No hits	–	No hits, AL382485 ($7e^{-30}$)
MtGmLs281	AJ311251	No hits	–	MtC00157 ($3.9e^{-34}$), TC60077 ($4.2e^{-34}$)
MtGmLs291	AJ311252	No hits	–	MtD05676 ($3.7e^{-79}$), TC72639 ($3.3e^{-79}$)
MtGmLs311	AJ311253	No hits	–	MtC30464 ($2.4e^{-44}$), TC69512 ($2.6e^{-44}$) weakly similar to transfactor-like protein
MtGmLs136	AJ311244	No hits	–	No hits, no hits
Fungal genes				
GmMtLs116	AJ31124	Peptidyl prolyl cis trans isomerase ($2e^{-36}$)	*Arabidopsis thaliana*	MtC00626 ($1.7e^{-33}$) probable peptidyl prolyl cis trans isomerase, TC73828 ($1.9e^{-73}$) peptidyl prolyl cis trans isomerase
GmMtLs221	AJ311223	Thioredoxin homologue ($4e^{-7}$)	*Naegleria fowleri*	MtC63121 ($1.8e^{-40}$), TC66256 ($4.6e^{-54}$)
GmMtLs119	AJ311256	No hits	–	No hits, TC62645 ($3.1e^{-194}$)
GmMtLs239	AJ311250	No hits	–	MtC00279 ($6.6e^{-55}$), TC61523 ($1.8e^{-56}$)
GmMtLs368	AJ311254	No hits	–	No hits, TC71889 ($6.5e^{-38}$)
GmMtLs17	AJ311241	No hits	–	No hits, no hits

Table 3 Sequences and annealing temperature (°C) of primers used to determine the origin of SSH ESTs

SSH ESTs to	Forward primer	Reverse primer	°C
GmMtLs17	AAAAGGGCAATGTCAGGG	AGTGTTTTGCACAGCA	55
GmMtLs116	TGGAAAGTCCATCTACGG	CATTAATCAGAGTTGACC	55
GmMtLs119	TAGATTGGGTACCACCCTACGG	GAGGTCCTAGTCCTCCAGAACC	60
GmMtLs221	CAACTCTGATGATCACGG	TTATTGATTGCCAAGAAC	55
GmMtLs239	CGCGTAACCCACTATTTAAGTG	TCCAACTTTTGCAACTTTACC	55
GmMtLs368	AACTCCAAAGTCAGGTGC	AATTAAGAGTCGATCA	55

Analysis of gene induction at early steps of mycorrhiza development

In order to analyse gene expression profiling in *M. truncatula* roots 5 dai with *G. mosseae* (fungal development limited to appressoria at the root surface), semi-quantitative RT-PCR and analysis of results were performed as described by Taylor and Harrier (2003), except for the cDNA synthesis and PCR reaction, which were performed in two different steps. Briefly, PCR products were quantified at different points of the amplification process to ensure that quantification was performed during the exponential phase. The level of plant *gapdh* transcripts was analysed in parallel in order to ensure equivalent reverse transcription and amplification. No change in *gapdh* gene expression was observed at this step of the symbiosis (Fig. 1; Table 4). Primers, annealing temperatures and the number of cycles used for quantification are indicated in Table 4. The plant genes encoding a nodulin 26-like protein and the ESTs MtGmLs164 and MtGmLs291 were significantly induced ($P<0.01$) during this early stage of root-fungal interaction (Fig. 4). The transcripts of the germin-like protein, MtGmLs11 and MtGmLs281 genes were only found in inoculated roots during the appressorium stage of *G. mosseae* development. The remaining plant genes were not activated at this stage.

Table 4 Primer sequences, annealing temperatures (°C) and number of cycles used in semi-quantitative RT PCR

Corresponding EST	Forward primer	Reverse primer	°C	Cycles
GAPDH	AGCAAGGACTGGAGAGGTGG	CATCGACGGTAGGCACACGG	60	26
Glutamine synthetase	CTCAGTTGGCATCTCTGCTGG	CATGTTAGATGATGGCCTCCTG	60	29
Nodulin 26-like	CCTGTTAGAACTCTAGGACC	CACTCTGGGTGATCTCACGC	60	29
PR 10	TGGATTGGCTGACACATTGG	ACAACCCAACCTTGATCTGG	60	26
Germin-like protein	GATGATACTGTCTTGGCTTTGG	GATGAAGAAAGCACCAACTCC	60	29
Putative wound-induced protein	CAACAGCAACAAGCAAAGCC	ATCAATTAGGACCCCAGCAGC	60	29
Glutathione-S-transferase	TTCTTGCACAATGACAACC	ACCATGCAGAATGAGGAATGG	57	29
MtGmLs11	CAAAGATTGCACCATCACCA	TTGCCTTTGGAACACTCACA	60	29
MtGmLs164	GTGTAGTCTTTAGCCTGTTGC	TGATTTGCATGTTTCACTCC	52	35
MtGmLs281	AATGGCTCCTGCACCAACACC	GGCTAGTCCTTTAATCAC	57	29
MtGmLs291	GATCACAGGCATCAAGGTGG	TTATGCTTGGAAGGTCTGC	57	35
MtGmLs311	CCTTTGCCCTCTCTTTCCG	GGTAGGTTGCTTCAGCCAGC	60	29
MtGmLs136	TTGCTAATGGAGATGGACC	TAACGTTGGAGTCAATGAGG	57	35

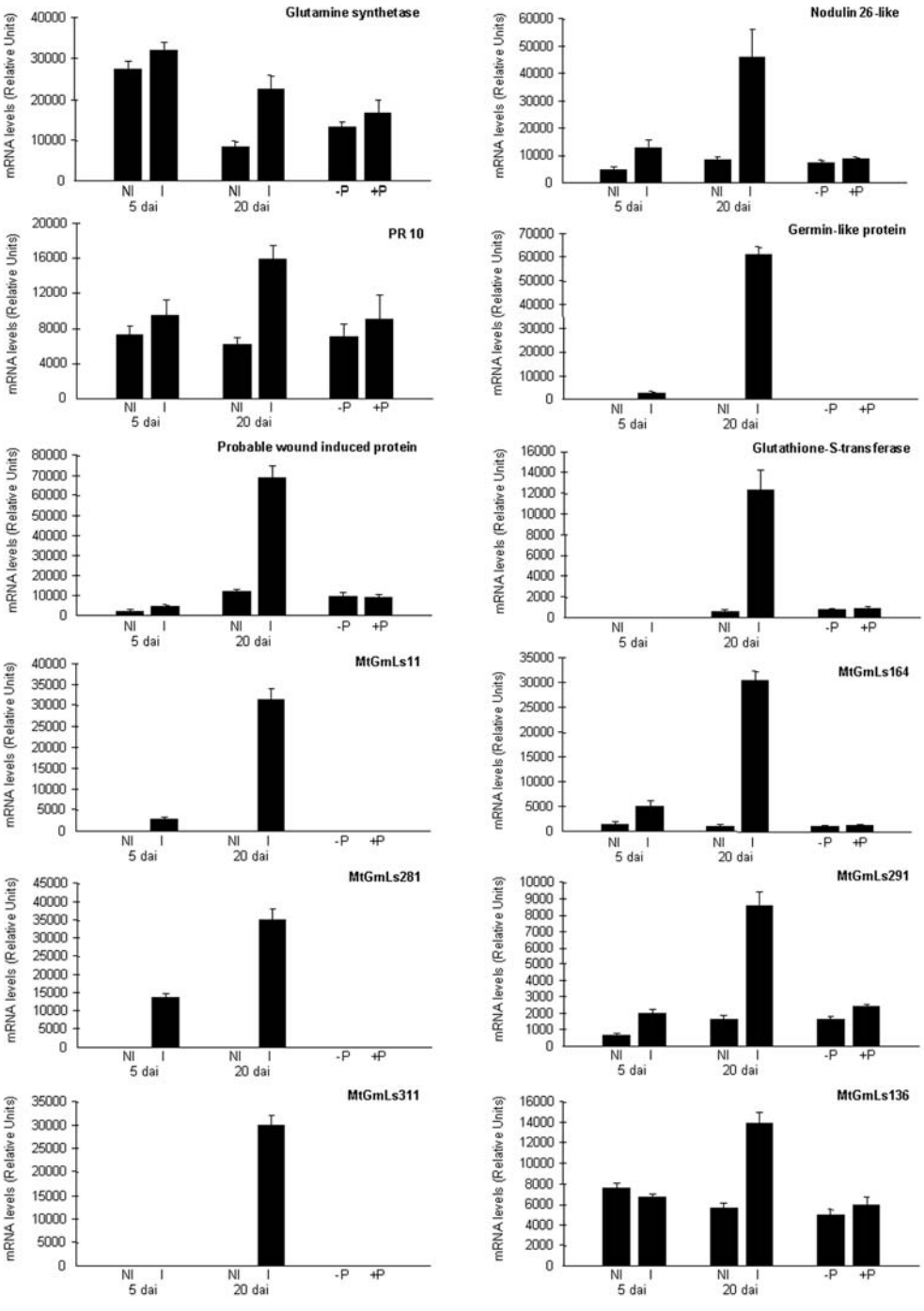

Fig. 4 Relative abundance of mRNA corresponding to 12 *M. truncatula* genes in roots 5 or 20 dai (*I*) with the fungus *G. mosseae* or non-inoculated (*NI*) or from plants fertilized (*+P*) or not (*–P*) with phosphate

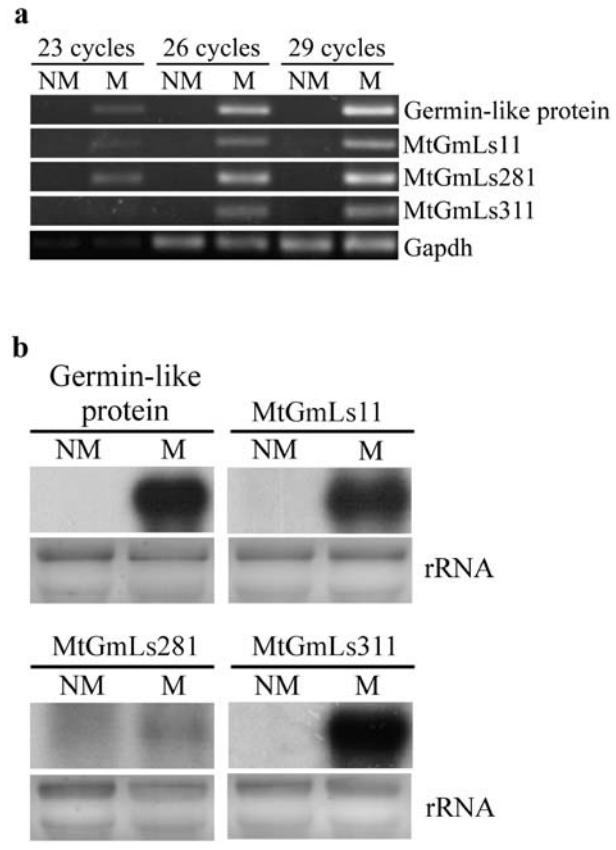

Fig. 5 Induced expression of four *M. truncatula* genes in mycorrhizal (20 dai) (*M*) and non-mycorrhizal (*NM*) roots, detected (**a**) by RT-PCR (control: gapdh) and (**b**) in Northern blots hybridized with ^{32}P-labelled EST probes (equal loading of RNA was verified by the rRNA)

Plant gene expression patterns in 3-week-old *M. truncatula* plants inoculated with *G. mosseae* or treated with phosphate

In order to confirm increased expression of plant genes corresponding to ESTs from the SSH library, semi-quantitative RT-PCR was performed using RNA from mycorrhizal roots (Table 1, experiments 3 and 4). Relative differences in mRNA accumulation between mycorrhizal and non-mycorrhizal roots confirmed significant enhancement ($P<0.01$) in the expression of eight plant genes during the symbiosis (Fig. 4). In addition, the transcripts of a germin-like protein, MtGmLs11 and MtGmLs281 genes, which were only detected in inoculated roots at the early stage of *G. mosseae* development, were also only found in roots presenting a well-established symbiosis (20 dai) and no PCR products were detected in non-inoculated roots (Fig. 4 and Fig. 5a). Likewise, the MtGmLs311 gene was also expressed in *G. mosseae* colonized roots at 20 dai (Fig. 4 and Fig. 5a). The specific expression of these four genes within arbuscular mycorrhiza was confirmed by Northern analysis (Fig. 5b). The level of *gapdh* transcripts was not modified in roots after phosphate treatment (Fig. 1; Table 4) and none of the 12 plant genes showed significant activation by phosphate (Fig. 4).

Expression profiling of fungal genes in the mycorrhizal symbiosis

Six genes of *G. mosseae* were identified by reverse Northern hybridization as being expressed in mycorrhizal roots 20 dai, of which five (peptidyl prolyl cis trans isomerase, thioredoxin homologue, GmMtLs119, GmMtLs239 and GmMtLs368) were also expressed during the early steps of fungal development 5 dai (Table 5). cDNA from non-colonized *M. truncatula* roots was used as a negative control. Expression of three of the fungal genes (peptidyl prolyl cis trans isomerase, thioredoxin homologue, GmMtLs119) was also common to external hyphae and spores of *G. mosseae*. Transcripts of one of the fungal genes without known similarities (GmMtLs17) were only detected in an established mycorrhiza (20 dai), indicating enhanced expression linked to development within the host tissues.

Discussion

Expression of plant and fungal genes during the AM symbiosis has mainly been investigated using pre-defined probes for known genes, or non-targeted approaches such as differential screening of cDNA libraries, differential RNA display analysis or classical subtractive hybridization (see for example Gianinazzi-Pearson 1996; Harrison 1999b; Martin-Laurent et al. 1997; Tahiri-Alaoui and Antoniw 1996; van Buuren et al. 1999). Recently, SSH has been used to analyse changes in gene expression of an AM fungus and of *M. truncatula* roots colonized by *G. intraradices* (Requena et al. 2002; Wulf et al. 2003). In the present study, SSH identified 12 plant and six fungal

Table 5 *Glomus mosseae* gene expression detected (+) or not (–) during different stages of fungal development

Fungal gene or EST	Spores	Mycorrhiza		Extraradical hyphae
		Early stage	Late stage	
Peptidyl prolyl cis trans isomerase (GmMtLs116)	+	+	+	+
Thioredoxin homologue (GmMtLs221)	+	+	+	+
GmMtLs17	–	–	+	–
GmMtLs119	+	+	+	+
GmMtLs239	–	+	+	–
GmMtLs368	–	+	+	–

genes that are expressed during interactions between *M. truncatula* and *G. mosseae*. All the plant genes and three of the fungal genes showed enhanced RNA accumulation within tissue of the AM symbiosis. Induction of the 12 plant genes was triggered by AM fungal colonization and was not the consequence of improved phosphate nutrition by the mycorrhiza. Four of these genes (germin-like protein, MtGmLs11, MtGmLs281 and MtGmLs311) were only expressed in mycorrhizal roots and not in non-inoculated roots, making them good candidates for molecular markers of mycorrhizal interactions. Only three of the plant genes (germin-like protein, glutathione-S-transferase, PR 10) have been reported previously to be activated in arbuscular mycorrhiza (Ruiz-Lozano et al. 1999; Wulf et al 2003). However, up-regulation of three *M. truncatula* genes and one fungal gene (glutathione-S-transferase, MtGmLs11, MtGmLs281, GmMtLs239) was indicated previously by electronic Northern analyses performed on accessible mycorrhizal and non-mycorrhizal cDNA library databases (Journet et al. 2002). Activation of these genes was confirmed experimentally in the present study. Induction of a further seven plant genes and expression of five fungal genes is described for the first time in the symbiotic interaction. Six of the *M. truncatula* genes are also activated in the early developmental stage of appressoria formation at the root surface, which suggests a role in mycorrhiza establishment and concords with the fact that mycorrhiza development is a dynamic process in which fungal appressoria are continually formed at the surface of growing roots. A further six plant genes, only induced in the arbuscular stages of mycorrhiza, could be involved in the development or functioning of the symbiosis.

Defence or stress-related gene expression has been well studied in arbuscular mycorrhiza (Gianinazzi-Pearson et al. 1996). As in *M. truncatula*, over-expression of a PR 10 gene occurs early in pea roots after inoculation with *G. mosseae*, as well as in a mycorrhiza-defective pea mutant (Ruiz-Lozano et al. 1999). Induction of a glutathione-S-transferase gene has previously been localized in potato root parenchymal cells colonized by a mycorrhizal fungus (Strittmatter et al. 1996). Transcription in *M. truncatula* mycorrhizal roots of the glutathione-S-transferase gene identified in the present study was also described by Wulf et al. (2003), and expression of the corresponding promoter was showed to be activated in mycorrhizal root tissue. In addition, it has been reported recently that, as for gene expression, the corresponding protein only accumulates in mycorrhizal roots (Bestel-Corre et al. 2002). Although this gene could be involved in defence responses to the symbiotic fungus, it may also be related to arbuscule senescence, since glutathione-S-transferase is considered to have protective functions (Dixit et al. 2001) and a tomato gene is induced during the process of cell death (Hoeberichts et al. 2001). The specific activation of a gene encoding a germin-like protein (GLP) only in *M. truncatula* roots colonized by *G. mosseae* also concords with results from the *M. truncatula-G. intraradices* interaction (Wulf et al. 2003). GLPs are coded by a large gene family in plants, and they can possess oxalate oxidase or superoxide dismutase activities (Woo et al. 2000). Some appear to be involved in responses to biotic or abiotic stresses in plants, whilst others may interact with membrane proteins in cell adhesion processes (Bernier and Berna 2001). In particular, the biotrophic powdery mildew fungus has been reported to trigger accumulation of germin mRNA after inoculation onto wheat seedlings (Hurkman and Tanaka 1996) and an enhanced oxalate oxidase activity in barley leaves (Zhou et al. 1998). The expression pattern of the GLP gene only in *G. mosseae*-inoculated *M. truncatula* roots makes it a good candidate for a marker gene of early and late stages of mycorrhizal symbiosis. On the other hand, the newly reported up-regulation of a gene encoding a wound-induced protein may reflect more general plant stress responses to AM fungal invasion.

The newly reported activation of an *M. truncatula* gene encoding glutamine synthetase in the established AM symbiosis, and not during early interactions, concords with reports that glutamine synthetase activity is higher in mycorrhizal than in non-mycorrhizal roots (Azcon and Tobar 1998; Cliquet and Stewart 1993). Likewise, this is the first time that enhanced RNA accumulation of a nodulin 26-like encoding gene has been reported in AM interactions, although a protein cross-reacting with nodulin 26 protein was detected immunologically in AM roots of soybean (Wyss et al. 1990). Nodulin 26 protein has been localized in the peribacteroid membrane of nodule symbiosomes, where it may act as an ion channel or be involved in malate transport across the symbiotic interface (Ouyang et al. 1991), and a similar role in transmembrane transport at the arbuscule interface in mycorrrhizal roots is feasible. Surprisingly, the nodulin 26-like gene is also activated when mycorrhizal interactions are limited to the early appressorium stage. However, nodulin 26 genes are members of an ancient conserved family and homologs exist that are expressed in vegetative parts of plants, such as root elongation and tip zones (Miao and Verma 1993). The presently identified *M. truncatula* gene requires further investigation before its role in the mycorrhizal symbiosis can be postulated.

Six ESTs without similarities were activated in early and/or late stages of *G. mosseae* development in *M. truncatula* roots. MtGmLs164 and MtGmLs291 were found in accessible non-symbiotic *M. truncatula* databases and cannot, therefore, be considered as marker genes of AM interactions. The four remaining genes without similarities, on the contrary, have never been reported to be activated in other biological systems and could represent genes novel to the mycorrhiza cell programme. Furthermore, MtGmLs11, MtGmLs281 and MtGmLs311 ESTs were found exclusively in accessible *M. truncatula* mycorrhizal cDNA library databases, which agrees with present results showing the expression of corresponding genes uniquely in *G. mosseae*-inoculated roots. The expression patterns of the genes encoding MtGmLs11 and MtGmLs281 make them potential mo-

lecular markers of early and late stages of mycorrhizal interactions, whilst the MtGmLs311 gene appears to be an indicator of a well established mycorrhizal symbiosis. PCR detection of transcripts of the plant genes encoding MtGmLs11, MtGmLs281 and MtGmLs311, together with GLP, could provide a reliable means of ensuring that an RNA population from mycorrhizal roots of *M. truncatula* is representative before proceeding to further transcriptomics studies. Determination of the expression pattern of these four genes during the nodulation symbiosis with *Sinorhizobium meliloti* or during a pathogen infection, for example with *Aphanomyces euteiches*, will be useful to determine whether these genes are specific to the mycorrhizal interaction.

Different targeted and non-targeted approaches have led to identification of AM fungal genes that are expressed in the symbiotic or asymbiotic steps of the life cycle (Franken and Requena 2000; Harrier 2001; Harrison 1999b). Some fungal genes expressed in mycorrhizal roots, such as *Gv1*, *Ginmyc1*, *Ginmyc2* or *Ginhb1*, have been identified (Burleigh and Harrison 1998; Delp et al. 2000), but their number remains low. In the present work, six new *G. mosseae* ESTs of genes expressed during the mycorrhizal symbiosis have been identified. This low number of fungal ESTs is probably due to the small amount of fungal RNA extractable from mycorrhizal roots. Maldonado-Mendoza et al. (2002) reported relative levels of fungal RNA reaching a maximum of only 5–12% in *M. truncatula* roots colonized by *G. versiforme*. Two of the *G. mosseae* genes show similarity to a peptidyl prolyl cis trans isomerase (PPIase) of the cyclophilin family and a thioredoxin homologue (TH). PPIases are believed to play a role in the folding of certain proteins by catalysing the cis-trans isomerization of X-Pro peptide bonds. Accumulation of transcripts of PPIase has already been described in plant responses to environmental stress (Godoy et al. 2000). TH participates in various redox reactions and has numerous functions in defence against oxidative stress, as well as in the control of growth and apoptosis (Arner and Holmgren 2000). Some transcription factors require thioredoxin reduction before binding DNA (Arner and Holmgren 2000). Expression of the genes encoding PPIase, TH and one without similarity (GmMtLs119) also occurred in extraradical hyphae, and two additional genes without similarity (GmMtLs239, GmMtLs368) were expressed during the early steps of root colonization. The latter two genes may be associated with appressorium differentiation by *G. mosseae* at the root surface and it would be interesting to characterize them in more detail, as appressoria are considered to be the critical event in the recognition of host tissues by fungi (Staples and Macko 1980) and are only induced by host roots (Giovannetti et al 1993, 1994; Giovannetti and Sbrana 1998). One of the fungal genes of unknown function, which was only expressed in the established mycorrhizal symbiosis (GmMtLs17), merits further investigation since it may be involved in the formation and/or the functioning of arbuscules.

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