Fungal Elicitation of Signal Transduction-Related Plant Genes Precedes Mycorrhiza Establishment and Requires the *dmi3* Gene in *Medicago truncatula*

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Suppressive subtractive hybridization and expressed sequence tag sequencing identified 29 plant genes which are upregulated during the appressorium stage of mycorrhiza establishment between *Medicago truncatula* J5 (Myc⁺) and *Glomus mosseae*. Eleven genes coding plant proteins with predicted functions in signal transduction, transcription, and translation were investigated in more detail for their relation to early events of symbiotic interactions. Expression profiling showed that the genes are activated not only from the appressorium stage up to the fully established symbiosis in the Myc⁺ genotype of *M. truncatula*, but also when the symbionts are not in direct cell contact, suggesting that diffusible fungal molecules (Myc factors) play a role in the induction of a signal-transduction pathway. Transcript accumulation in roots of a mycorrhiza-defective Myc⁻ *dmi3* mutant of *M. truncatula* is not modified by appressorium formation or diffusible fungal molecules, indicating that the signal transduction pathway is required for a successful *G. mosseae*–*M. truncatula* interaction leading to symbiosis development. The symbiotic nodulating bacterium *Sinorhizobium meliloti* does not activate the 11 genes, which supposes early discrimination by plant roots between the microbial symbionts.

Additional keywords: signaling pathway.

Terrestrial plants have developed numerous strategies to cope with the diverse biotic and abiotic challenges that they have met with during evolution. One of the most successful strategies is their ability to establish reciprocally beneficial symbiotic relationships with microorganisms (Gianinazzi-Pearson 1996), of which the most widespread is represented by arbuscular mycorrhizal (AM) associations between Glomeromycota fungi and root systems (Schüssler et al. 2001; Walker and Trappe 1993). Arbuscular mycorrhizas are instrumental in the survival and fitness of many plant taxa in diverse ecosystems through their function in the efficient exploitation of soil mineral resources and their bioprotective role against a number of common soilborne pathogens (Barea et al. 2002). The development of this type of symbiosis requires significant morphophysiological alterations in both plant and fungal cells that are assumed to be coordinated via a reciprocal exchange of signals (Liu et al. 2003). The colonization of root tissues by an

AM fungus follows a well-defined series of morphogenetic events which begin with hyphal branching and formation of an appressorium at the root surface (Giovannetti et al. 1994). These culminate in the intense proliferation of mycelium within the cortical parenchyma, where differentiation of specialized, highly branched, arbuscules is induced within host cells (Gianinazzi-Pearson 1996).

Little is known of molecular interactions prior to and during initial contact between AM fungi and plant roots, mainly due to difficulties in synchronizing developmental events in the two symbionts in order to dissect gene expression patterns and correlate them with these early stages of the symbiosis. AM fungi show limited development during asymbiotic spore germination. In *Glomus mosseae* (Nicol. & Gerd.), this is associated with expression of a GTPase-encoding gene which may be involved in programmed cell death of hyphae in the absence of a host signal (Requena et al. 2002). The perception of specific plant signals by AM fungi is indicated by gene activation in *Gigaspora rosea* (Nicolson & Schenck) that is elicited by host root exudates during the developmental switch from spore germination to extensive hyphal branching (Tamasloukht et al. 2003), and by the induction of an H⁺-ATPase gene *GmHA5* in *Glomus mosseae* upon appressorium formation on host roots (Requena et al. 2003). In this context, there exists evidence that appressoria may form in response to a plant signal endogenous to the root epidermal cell wall (Nagahashi and Douds 1997).

Plant gene expression that has been monitored during the appressorium stage of the AM symbiosis is mainly related to defense responses. Several studies on *Medicago truncatula* Gaertn. have demonstrated that plant defense genes generally show weak and transient activation in early root interactions with AM fungi (Bonanomi et al. 2001; Harrison 1999). In tobacco, a transient increase in catalase and ascorbate peroxidase activities appeared with the formation of the first appressoria by *G. mosseae* on roots (Blilou et al. 2000), and the expression of one defense-related gene in pea (*pl 206*) was correlated with appressorium formation by this fungus (Ruiz-Lozano et al. 1999). Such activation of plant defense genes during the early stages of mycorrhizal interactions gave first indications for the existence of fungal signal molecules.

More direct evidence that AM fungi can induce a localized gene response to appressorium in host roots has been provided by studies on *MtENOD11*-driven *gusA* expression in *M. truncatula*. Epidermal cells in the immediate vicinity of appressoria formed by *Gigaspora* spp. show a transient low level of transgene activity which disappears with the appearance of a

strong gene expression in cells where arbuscules develop (Chabaud et al. 2002). It subsequently was shown that a diffusible AM fungal ("Myc") factor could induce strong *MtENOD11*-gusA expression, primarily in root cortical tissues without cell contact between the symbionts (Kosuta et al. 2003).

AM formation and nodulation are commonly controlled by a number of genes in *M. truncatula* (*DMI1*, *DMI2*, and *DMI3*), pea (*sym8*, *sym19*, and *sym9/30*), and *Lotus japonicus* (*LjsymRK*) (Albrecht et al. 1999; Catoira et al. 2000; Duc et al. 1989; Endre et al. 2002; Gianinazzi-Pearson 1996; Stracke et al. 2002). The genes *DMI2* and *LjsymRK* have been identified as coding a receptor-like kinase (Endre et al. 2002; Stracke et al. 2002). It recently has been reported that the *DMI3* gene is highly similar to a calcium- and calmodulin-dependent protein kinase, while the *DMI1* gene codes a novel protein showing low similarity to a ligand-gated cation channel (Ané et al. 2004; Lévy et al. 2004; Mitra et al. 2004). Plants mutated for these genes are unable to establish a functional association with rhizobia and AM fungi (Nod-/Myc⁻ phenotypes), but formation of appressoria by the latter does occur on their roots without hyphal penetration (Calantzis et al. 2001; Catoira et al. 2000; Sagan et al. 1995; Wegel et al. 1998). The *MtENOD11* gene is activated by the diffusible AM fungal factor in roots of Myc⁻ *dmi1*, *dmi2*, and *dmi3* mutants, suggesting that a DMI-independent signal transduction pathway is involved in this response (Kosuta et al. 2003). However, contact-driven expression of this gene is absent from epidermal root cells of a Myc⁻ *dmi2* mutant associated with appressoria, suggesting that two Myc signals may activate *MtENOD11*, one that requires contact with the plant and is DMI dependent, and one that does not require contact and is DMI independent. Likewise, we recently have found six other appressorium-induced *M. truncatula* genes that are DMI dependent because they are not activated during initial fungal contact with root cells in a Myc⁻ *dmi3* mutant (Breckenmacher 2003; Breckenmacher et al. 2004).

To further elucidate the molecular dialogue which is implemented during early events leading to AM development, we have identified genes expressed in root systems synchronized for the appressorium stage of interactions between *M. truncatula* and *G. mosseae*. Studies were focused on pathways potentially related to symbiotic fungal signal perception by *M. truncatula* in Myc⁺ and Myc⁻ genotypes. We present data showing

that activation of plant genes related to signal transduction and translation prior to and during symbiont cell contact is associated with successful root colonization.

RESULTS

Microbe development and plant growth.

G. mosseae consistently developed the first appressoria on the root surface of the *M. truncatula* J5 genotype 3 days after inoculation (dai) and reached an average of 4.2 appressoria per cm root 5 dai. Fungal development was slower on roots of the Myc⁻ TRV25 mutant and needed 7 dai to reach a similar level of appressorium development (3.7 appressoria/cm of root). Root penetration never was observed after trypan blue coloration in different experiments at these time points in either genotype (Fig. 1A and B). First arbuscules appeared 6 to 7 dai in roots of *M. truncatula* J5 and the mycorrhizal symbiosis was fully established in *M. truncatula* J5 plants at 10 dai, with 84% of the root system colonized and 31% containing arbuscules (Fig. 1C). At this time, an average of 3.1 appressoria/cm of root had developed. In all cases, no contamination by other microorganisms was observed. Root colonization of *M. truncatula* J5 plants by *S. meliloti* was evaluated as being 2×10^8 bacteria/root system at 4 dai and there was no significant effect of inoculation on root or shoot growth. No nodules had developed at this time.

Shoot fresh biomass of the *G. mosseae*-inoculated J5 genotype, with (average 30.7 mg) or without (average 40 mg) root contact, was significantly ($P < 0.0001$) higher than noninoculated plants (22.6 and 30 mg, respectively) at 5 dai. Root fresh weights of *M. truncatula* J5 plants 5 dai, with (32.3 mg) or without (37 mg) contact by *G. mosseae*, also were significantly ($P < 0.05$) greater than those of noninoculated control plants (26.2 and 25.7 mg, respectively). These effects of *G. mosseae* inoculation on plant growth were not observed for the TRV25 genotype. When the *M. truncatula* J5 genotype plants reached 10 dai, the shoot fresh biomass was significantly ($P < 0.0001$) different between noninoculated (average 43 mg) and inoculated (average 45.3 mg) plants. On the contrary, root fresh weights were not significantly different between treatments at 10 dai by *G. mosseae*.

Identification of appressorium-induced genes and proteins.

The subtractive suppressive hybridization (SSH) library (*M. truncatula*-*G. mosseae* early stage [MtGmEs] interaction) of 1,444 clones (average insert size = 570 bp) was screened with cDNA probes from noninoculated and *G. mosseae*-inoculated *M. truncatula* J5 roots (5 dai) of plants different from those used to construct the SSH library (average 3.8 appressoria/cm of root). Polymerase chain reaction (PCR) (with primers corresponding to selected expressed sequence tags [ESTs]) on the RNA samples before reverse transcription gave no amplification signals, confirming that there was no contamination by genomic DNA (data not shown). Reverse Northern hybridization signals were normalized using the *Mtgap1* gene. ESTs corresponding to genes with at least 2.5-fold increases in transcript accumulation in three experimental repeats of inoculated compared with control roots were sequence analyzed. Analysis of 900 clones gave 29 genes which consistently were upregulated in *G. mosseae*-inoculated *M. truncatula* J5 roots (Table 1). Sequences for which no redundancy was detected were designated as MtGmEs and deposited in the EMBL databases (accession numbers AJ621847 to AJ621875). All of these genes presented a basal expression in noninoculated roots and had E values showing significant similarities only to plant genes. The most represented gene categories coded for proteins related to primary

Fig. 1. *Medicago truncatula*-*Glomus mosseae* interactions. **A**, Appressoria (ap) on a root of the Myc⁺ genotype J5 5 days after inoculation (dai). Bar = 25 μ m. **B**, Appressoria (ap) on a root of the Myc⁻ mutant TRV25 7 dai. Bar = 30 μ m. **C**, Appressorium (ap) and intraradical mycelium (im) in a mycorrhizal root of the genotype J5 10 dai. Bar = 100 μ m. **D**, Spore (sp) and hyphal network (h) developed on a membrane after 2 weeks. Bar = 0.4 mm. **E**, Reverse-transcription polymerase chain reaction detection of large subunit ribosomal RNA of *G. mosseae* from *M. truncatula* J5 roots grown without inoculation (lane 1), with germinated spores separated by a membrane (lane 2), 5 dai by *G. mosseae* (lane 3), and 10 dai by *G. mosseae* (lane 4). The fifth lane corresponds to the water control.

metabolism ($n = 6$), protein synthesis ($n = 7$), signal transduction ($n = 4$), or unknown functions ($n = 5$). None of these genes previously have been reported to be expressed in mycorrhizal interactions. For the six genes of the primary metabolism category, two corresponded to *Arabidopsis thaliana* L. Heynh sequences encoding an F1F0-ATPase inhibitor-like protein and a methylenetetrahydrofolate reductase, two to *Nicotiana tabacum* L. sequences with diacylglycerol acylCoA acyltransferase and an isopentenyl diphosphate isomerase functions, one to a ferredoxin nitrite reductase gene of soybean, and one to a GDP-mannose pyrophosphorylase gene of *Solanum tuberosum* L. (Table 1). The four ESTs of the signal transduction category matched genes encoding a tomato calcium lipid binding protein, an *A. thaliana* MAP kinase, a cotton fiber annexin, and an *L. japonicus* nitrate reductase. In the category of protein synthesis, three matching sequences originated from *A. thaliana* (eukaryotic initiation factor 4F, 40S ribosomal protein S3, and 60S ribosomal protein L7), two from *Castanea sativa* Mill. (60S ribosomal protein L18a), one from potato (small nuclear ribonucleoprotein UA1), one from *N. tabacum* (eukaryotic initiation factor 4A), and one from *N. paniculata* L. (elongation factor 1- α).

Gene expression in wild-type *M. truncatula* J5 and the Myc⁻ *dmi3* mutant TRV25.

Following the above analyses, four genes related to signal transduction (annexin, calcium lipid binding protein, MAP kinase, and nitrate reductase), one to transcription (small nuclear ribonucleoprotein UA1), and six to translation events (two eukaryotic initiation factors, three ribosomal proteins, and one elongation factor 1- α) were selected for more detailed investigation. Expression profiling was performed by semi-quantitative reverse transcription (RT)-PCR (Burleigh 2001) using primers deduced from the EST sequences corresponding

to the 11 genes and the number of cycles given in Table 2. Contrary to Northern analyses, semi-quantitative RT-PCR not only enables specific detection of a gene family member but also is sufficiently sensitive to detect transcripts in limited amounts of tissue like those interacting with appressoria. As above, *Mtgap1* gene expression was chosen as the reference. Roots from new sets of *G. mosseae*-inoculated and noninoculated *M. truncatula* plants were used. RNA was extracted and checked for the absence of DNA contamination. Representative RT-PCR profiles are shown in Figure 2. Significant activation ($P < 0.05$) was confirmed in roots of the *M. truncatula* J5 genotype 5 dai by *G. mosseae* from three independent inoculation experiments (average, 4 appressoria/cm of root) for all the genes (Fig. 3A). None of the 11 plant genes were significantly upregulated by *G. mosseae* appressorium formation on roots (average, 3.7 appressorium/cm of root) of the *M. truncatula* *dmi3* mutant 7 dai (Fig. 3B), where the fungus cannot penetrate inside the root tissues. The 11 genes activated in *M. truncatula* roots at the appressorium stage likewise were upregulated at 10 dai when *G. mosseae* had proliferated further along and colonized roots (Fig. 2).

Gene expression prior to fungal-root contact.

Transcripts of the *M. truncatula* genes related to signal transduction, transcription, and translation also were monitored before contact between root and fungal cells. For this, spores of *G. mosseae* pregerminated between nitrocellulose membranes were placed on *M. truncatula* roots (J5 genotype or *dmi3* mutant) to physically separate fungal and plant tissues but allow diffusion of molecules between them. On average, 50% of spores germinated in the presence of either plant genotype and produced a ramified hyphal network on the membranes (Fig. 1D). Expression profiling of the 11 *M. truncatula* genes showed that they were significantly upregulated in root sys-

Table 1. Sequence similarities at protein levels for expressed sequence tags of ≥ 2.5 -fold upregulated genes in *Medicago truncatula* J5 roots 5 days after inoculation with *Glomus mosseae*

MtGmEs clone ^a	Accession number	Matching predicted protein ^b	TIGR <i>M. truncatula</i> gene index	E value (Blast X)
1	AJ621848	Similar to <i>Arabidopsis thaliana</i> chromosome 5— <i>A. thaliana</i>	TC77198	1.6 e ⁻³⁴
9	AJ621847	Ca ²⁺ lipid binding (CLB1) protein— <i>Lycopersicon esculentum</i>	TC78567	1.1 e ⁻⁸²
12	AJ621849	40S ribosomal protein S3 (40S RP S3)— <i>A. thaliana</i>	TC85147	1.1 e ⁻²²
14	AJ621850	Diacylglycerol acylCoA acyltransferase— <i>Nicotiana tabacum</i>	BQ148998	7.1 e ⁻⁴⁷
108	AJ621851	HMG1/Y protein—Garden pea	TC77788	4.6 e ⁻¹¹³
133	AJ621852	MAP kinase (MAPK)— <i>A. thaliana</i>	TC87038	6.3 e ⁻¹¹⁴
160	AJ621853	Isopentenyl diphosphate isomerase 1— <i>N. tabacum</i>	TC77413	3.6 e ⁻¹¹³
330	AJ621854	Annexin—Upland cotton	TC85793	1.1 e ⁻⁷⁸
350	AJ621855	F1F0-ATPase inhibitor-like protein— <i>A. thaliana</i>	TC86884	9.4 e ⁻⁹²
367	AJ621856	60 ribosomal protein L7 (60S RP L7)— <i>A. thaliana</i>	TC77462	8.5 e ⁻²⁴
376	AJ621857	Putative pollen germination related protein— <i>Oryza sativa</i>	TC77426	3.0 e ⁻⁸⁹
445	AJ621858	Methylene tetrahydrofolate reductase— <i>A. thaliana</i>	TC85756	2.3 e ⁻¹²⁰
457	AJ621859	Hs1pro-1 homolog— <i>Pisum sativum</i>	TC76759	2.1 e ⁻⁸⁵
470	AJ621860	Ribosomal protein 18a— <i>Castanea sativa</i>	TC76395	6.9 e ⁻³⁸
642	AJ621861	Ferredoxin nitrite reductase—soybean	TC77277	2.9 e ⁻¹⁶³
644	AJ621862	Weakly similar to receptor-like protein kinase homolog RK20-1— <i>Phaseolus vulgaris</i>	TC85561	2.8 e ⁻³⁶
645	AJ621863	Eukaryotic initiation factor 4F (eIF4F)— <i>A. thaliana</i>	TC86593	2.4 e ⁻³³
670	AJ621864	ADR6—soybean	TC86246	2.3 e ⁻⁶⁷
762	AJ621865	60S ribosomal protein L18a (60S RP L18a)— <i>C. sativa</i>	TC76395	1.2 e ⁻³¹
768	AJ621866	Eukaryotic initiation factor 4A (eIF4A)— <i>N. tabacum</i>	TC76958	5.6 e ⁻¹²⁶
770	AJ621867	Nitrate reductase (NR)— <i>Lotus japonicus</i>	TC78214	8.3 e ⁻⁸⁷
771	AJ621868	Small nuclear ribonucleoprotein UA1 (SnRNP UA1)— <i>Solanum tuberosum</i>	TC78767	8.8 e ⁻⁴⁶
773	AJ621869	GDP-mannose pyrophosphorylase— <i>S. tuberosum</i>	TC77044	3.7 e ⁻³⁹
774	AJ621870	Expansin— <i>Prunus cerasus</i>	TC78682	5.4 e ⁻⁷²
775	AJ621871	Extensin-like protein S3— <i>M. sativa</i>	TC78495	9.8 e ⁻⁴⁴
776	AJ621872	Cyclin B— <i>P. sativum</i>	AL377325	2.4 e ⁻⁴⁵
777	AJ621873	Unnamed protein product— <i>L. esculentum</i>	TC85735	1.1 e ⁻⁴⁴
890	AJ621874	Elongation factor-1 alpha (EF 1 α)— <i>N. paniculata</i>	TC85175	5.3 e ⁻⁰⁹
899	AJ621875	npp	...	...

^a MtGmEs = *M. truncatula*-*G. mosseae* early stage.

^b npp = No predicted protein.

tems of the J5 genotype after 5 days in the presence of the fungus, compared with control roots (Fig. 3C), except for one of them (annexin), for which activation was consistently, but not significantly, observed due to some variation between samples. On the contrary, none of the *M. truncatula* genes were upregulated when the fungus was separated from roots of the *dmi3* Myc⁻ mutant (Fig. 2D). No hyphal contact with *M. truncatula* roots was ever observed after extensive microscopic examination of root systems stained with trypan blue. This was con-

firmed by diagnostic RT-PCR to detect large subunit (LSU) ribosomal (r)RNA of *G. mosseae* which gave no signals in cDNA samples from control roots or root systems separated by a membrane from the fungus, while a PCR product was detected from roots at the appressoria stage (5 dai) and in mycorrhizal roots (10 dai) (Fig. 1E).

Gene responses to *S. meliloti*.

Expression of the 11 genes related to signal transduction, transcription, and translation was analyzed in *M. truncatula* 4 dai with *S. meliloti* in order to investigate possible common expression during early stages of the nodulation and mycorrhizal symbioses. A significant increase in transcripts was detected for only one gene corresponding to the SnRNP UA1 protein (Fig. 4). The expression of the other genes was not enhanced by inoculation of the bacteria, and expression of the nitrate reductase gene was significantly downregulated in the presence of *S. meliloti* (Fig. 4).

DISCUSSION

The lack of information concerning molecular interactions prior to and during initial cell-to-cell contact between AM fungi and host roots prompted the present investigation of gene responses to appressorium formation by *G. mosseae* at the root surface of *M. truncatula*. Among the 29 activated *M. truncatula* gene that are reported for the first time during these early events, 11 are related to signal transduction, transcription, and translation. Results indicate that these 11 genes form part of a molecular pathway which is associated with symbiosis establishment. This conclusion is inferred from the observation that expression of the corresponding genes is not enhanced when *G. mosseae* develops appressoria on roots of the Myc⁻ *dmi3* mutant, where fungal development cannot proceed beyond the epidermis. Moreover, the signal transduction-related set of *M. truncatula* genes also are induced when roots of the wild-type genotype are separated from *G. mosseae* hyphae by a physical barrier, demonstrating that a diffusible fungal factor perceived by the host tissues is implicated. Similar observations on *MtENOD11* activation by different AM fungi previously has led to the hypothesis of a putative Myc factor which could play an analogous role in AM signal mechanisms to Nod factors in

Fig. 2. Representative reverse-transcription polymerase chain reaction profiles for four genes of signal transduction, one of transcription, and six of translation in i) the Myc⁺ *Medicago truncatula* J5 genotype at 5 and 10 days after inoculation (dai) by *Glomus mosseae*, ii) the Myc⁻ TRV25 genotype 7 dai, and iii) the Myc⁺ J5 and Myc⁻ TRV 25 genotypes separated from germinated spores and hyphae of *G. mosseae* roots by a nitrocellulose membrane. NI: noninoculated; I: inoculated. Signal transduction genes: Annexin, CLB1 protein (Ca²⁺ lipid binding protein), MAPK (MAP kinase), NR (nitrate reductase); transcription gene: SnRNP UA1 (small nuclear ribonucleoprotein UA1); translation genes: eIF-4A (eukaryotic initiation factor 4A), eIF-4F (eukaryotic initiation factor 4F), 40S RP S3 (40S ribosomal protein S3), 60S RP L7 (60S ribosomal protein L7), 60S RP L18a (60S ribosomal protein L18a), and EF-1α (elongation factor 1-alpha). *Mtgap1* (glyceraldehydes phosphate dehydrogenase gene).

Table 2. Oligonucleotide primer sequences used in semi-quantitative reverse-transcription polymerase chain reaction analyses

MtGmEs ^a	Similarity ^b	Primer sequences	Number of cycles
330	Annexin	GAAGTCTCCGGTTGTGTGGT GGACATCGAGCAATCAGGTT	27
9	CLB1 protein	GCTGCTGTCCATCGCTTGGATA CGGCAGCTTTCAGTTTCTTC	25
133	MAPK	CAATGCAATTGCAAAG GGCAGTGTCTTTTGGTGT	27
770	NR	CTTTAAACCCGCGTGAGAAA TCATCTACGCTGCTTGTGG	27
771	SnRNP UA1	TGCAAGTAATGGTGGCTCAA AAAGCATTTCGAGCATCTG	25
768	EIF-4A	TTTGGAAGGAAAGGTGTTGC AGTTCAGCAACATGGGAAGG	27
645	EIF-4F	CAAGCACCAGGGCACTATTC CTGGAGGACGTGGACAATCT	25
12	40S RP S3	CATCCGGACAGCCTGTTAAG CTCCTCCCTTTGGAGTGTGA	27
367	60S RP L7	TCCATCAGTTGGCTTCACAA ATCGCAACAGGAGATTGACC	27
762	60S RP L18a	CGTTTTGGCCAAGTATTGCT TGATGCTGAATTTGGTGGAA	27
890	EF-1α	TGGACATGTCGACTCTGGAA TGTTTCATCTCAGCAGCTTCCT	22

^a MtGmEs = *Medicago truncatula*-*Glomus mosseae* early stage.

^b MAPK = MAP kinase, NR = nitrate reductase, EIF = eukaryotic initiation factor.

the rhizobial symbiosis (Kosuta et al. 2003). In contrast to the *MtENOD11* gene which is consistently activated by AM fungal molecules in roots of *Myc⁻ dmi1*, *dmi2*, and *dmi3* mutants of *M. truncatula* (Kosuta et al. 2003), the diffusible factor from *G. mosseae* does not trigger activation of the signal transduction-related genes in the *dmi3* mutant. This is consistent with the specific sensing of a *Myc* factor by host roots and suggests that the *dmi3* gene is situated upstream in the cascade of events involved in perception of the fungal signal molecule or molecules.

Activation of two genes encoding a calcium lipid binding protein and an annexin during early *M. truncatula* J5–*G. mosseae* interactions could reflect the involvement of a calcium-modulated signaling pathway in the establishment of the AM symbiosis. This is further supposed from the similarity of *dmi3* to genes encoding calcium- and calmodulin-dependant protein kinases (Lévy et al. 2004; Mitra et al. 2004). Calcium is

an intracellular messenger coordinating responses to numerous developmental cues and environmental challenges (White et al. 2003). Annexins belong to a family of calcium-binding proteins which are known to participate in a variety of Ca^{2+} -regulated cellular processes (Morgan and Pilar Fernandez 1997). A member of the annexin family, *MtAnn1*, is induced by Nod factors during the early stages of nodulation, where it may play a role in cell cycle reactivation or cytoskeleton rearrangements during root–rhizobia interactions (Carvalho-Niebel et al. 2002). Moreover, it has been shown that calcium spiking is observed minutes after the addition of Nod factors, suggesting a role for Ca^{2+} as a secondary messenger in signal transduction of the Nod factor (Felle et al. 1999). In *dmi1*, *dmi2*, and *dmi3* mutants of *M. truncatula*, which are unable to establish nodule or mycorrhizal symbioses, the calcium spiking response to Nod factors is conserved only in plants mutated for the *dmi3* gene, indicating that it is downstream of the other two genes in

Fig. 3. Relative abundance of transcripts (semi-quantitative reverse-transcription polymerase chain reaction) of 11 *Medicago truncatula* genes. **A**, *Glomus mosseae*-inoculated (■) and noninoculated (□) roots of the *Myc⁺* genotype J5 5 days after inoculation (dai). **B**, *G. mosseae*-inoculated (■) and noninoculated (□) roots of the *Myc⁻* TRV25 genotype 7 dai. **C**, *Myc⁺* *M. truncatula* J5 grown 5 days in the presence (■) or absence (□) of germinated spores and hyphae of *G. mosseae* separated from roots by a nitrocellulose membrane. **D**, *Myc⁻* *M. truncatula* TRV25 grown 7 days in the presence (■) or absence (□) of germinated spores and hyphae of *G. mosseae* separated from roots by a nitrocellulose membrane. Bars represent standard errors of means. Signal transduction genes: Annexin, CLB1 protein (Ca^{2+} lipid binding protein), MAPK (MAP kinase), NR (nitrate reductase); transcription gene: SnRNP UA1 (small nuclear ribonucleoprotein UA1); translation genes: eIF-4A (eukaryotic initiation factor 4A), eIF-4F (eukaryotic initiation factor 4F), 40S RP S3 (40S ribosomal protein S3), 60S RP L7 (60S ribosomal protein L7), 60S RP L18a (60S ribosomal protein L18a), and EF-1α (elongation factor 1-α).

a signaling cascade (Catoira et al. 2000; Wais et al. 2000). The existence of mutants in *M. truncatula* and other legumes which are defective for nodulation and mycorrhization supposes common steps between the two root symbioses (Albrecht et al. 1999). However, Kosuta and associates (2003) provided evidence that the *MtENOD11* gene responding to diffusible signals from both rhizobia and AM fungi is differentially regulated in the two symbioses and suggested that signal transduction in response to Nod and Myc factors occurs via different pathways. This hypothesis also is inferred from the present observations in that the signal transduction-related *M. truncatula* pathway elicited by *G. mosseae* is not activated in roots inoculated for 4 days by *S. meliloti*. Furthermore, the fact that the mycorrhiza-related signaling pathway is inactivated by mutation of the *dmi3* gene lends support to a role of the *dmi3* gene product in distinguishing the Myc factor from Nod factors, and in specific activation of the signal transduction pathway leading to mycorrhiza development.

The induction of a *M. truncatula* mitogen-activated protein (MAP) kinase gene by *G. mosseae* prior to and during appressorium formation on roots is consistent with the role of MAP kinases in the transduction of extracellular signals to intracellular targets in eukaryotes (Suzuki 2002). Distinct MAP kinase pathways are implicated in a wide variety of biological processes and, in plants, they are considered to play a role in signaling responses to abiotic stresses, pathogens, and plant hormones (Jonak et al. 1999). After translocation into the nucleus, they can activate sets of genes through phosphorylation of specific transcription factors. They have been well studied in defense reactions (Jonak et al. 1999; Zhang et al. 2001), and two MAP kinases involved in defense response and phosphorylating myelin basic protein on serine or threonine residues have been shown to be dependent on Ca^{2+} influx into cells (Lebrun-Garcia et al. 1998). The identity and the number of MAP

kinases, their sustained activation, and their regulation in general may be determinant points directing downstream events in signal transduction pathways (Lebrun-Garcia et al. 1998). This may be relevant to arbuscular mycorrhiza interactions because two other MAP kinases, showing no homology to the gene reported here, have been reported to be upregulated during early root colonization events by *Glomus versiforme* (Karsten) Berch in *M. truncatula* (Liu et al. 2003).

Although nitrate reductase is a key enzyme of nitrate assimilation in higher plants through its conversion of nitrate to nitrite, it also is implicated in the generation of NO from nitrite (Neill et al. 2003). The nitrate reductase gene of *M. truncatula* is activated before symbiosis establishment by *G. mosseae*; therefore, it may be related to this second function in the early root-fungal interactions. NO, which is recognized to be a widespread signaling molecule in mammal cells, is now considered as an endogenous maturation- and senescence-regulating factor in higher plants (Wendehenne et al. 2001). It is associated with several physiological responses in plants, including plant defense responses, programmed cell death, stomatal closure, or root growth. The mobile nature of NO and its chemical reactivity with various cell targets make it a potentially important molecule in cell responses. The downstream effects of NO may be induced directly by its interactions with, for example, ion channel proteins or proteins that regulate gene expression, or indirectly by interactions with signaling proteins such as protein kinases or with secondary messenger-generative enzymes (Neill et al. 2003). NO appears to act through cGMP and cADPR to activate intracellular Ca^{2+} -permeable channels, and also plays a role in elevating free cytosolic Ca^{2+} (Wendehenne et al. 2004). This is particularly relevant in relation to the fact that *DMI3* encodes a Ca^{2+} - and calmodulin-dependent protein kinase in *M. truncatula*.

The concomitant activation by *G. mosseae* in *M. truncatula* J5 roots of a small nuclear ribonucleoprotein UA1 gene with a role in mRNA splicing is suggestive of enhanced transcriptional activity. The induction of ribosomal genes along with initiation and elongation factors is coherent with subsequent translational events leading to de novo protein synthesis and cell activation in *Myc*⁺ roots responding to AM fungal signals. The fact that none of these genes respond to appressorium development in the *Myc*⁻ genotype supports the hypothesis that they are active in transcription and translation events downstream of those associated with the signal transduction pathway or pathways elicited by *G. mosseae*. Cell activation by appressorium development on *Myc*⁺ roots is seen in the other ESTs identified in the SSH library and in coding for proteins involved in cell cycle machinery (cyclin B), lipid metabolism (diacylglycerol acylCoA acyltransferase and isopentenyl diphosphate isomerase), or cell wall properties (extensin and expansin).

In conclusion, most studies in the past have focused on genes related to the arbuscular stage of mycorrhizal interactions. In the present study, we have provided evidence that a signal transduction and translation pathway leading to the establishment of the mycorrhizal symbiosis is activated in roots by AM fungal molecules, and that plant gene activation occurs without contact between the symbionts. The lack of activation of this molecular pathway by *S. meliloti* points to cell processes which discriminate at an early point between mycorrhizal and nodulating root symbionts. The identification of signal transduction-related plant genes responding to AM fungal molecules opens new perspectives for deciphering the molecular dialogue which is involved in the recognition process necessary for symbiosis establishment, and for pinpointing receptors at the host cell surface which specifically sense the AM fungal signals.

Fig 4. Relative abundance of transcripts (semi quantitative reverse-transcription polymerase chain reaction) of 11 *Medicago truncatula* genes from *Sinorhizobium meliloti*-inoculated (■) and noninoculated (□) roots of the *Myc*⁺ genotype J5 4 days after inoculation. Bars represent standard errors of means. Signal transduction genes: Annexin, CLB1 protein (Ca^{2+} lipid binding protein), MAPK (MAP kinase), NR (nitrate reductase); transcription gene: SnRNP UA1 (small nuclear ribonucleoprotein UA1); translation genes: eIF-4A (eukaryotic initiation factor 4A), eIF-4F (eukaryotic initiation factor 4F), 40S RP S3 (40S ribosomal protein S3), 60S RP L7 (60S ribosomal protein L7), 60S RP L18a (60S ribosomal protein L18a), and EF-1α (elongation factor 1-α).

MATERIALS AND METHODS

Plant growth and inoculation.

Seed of *M. truncatula* Gaertn. cv. Jemalong, line J5, and the *Myo⁻ dmi3* mutant TRV25 (provided by G. Duc, INRA-Dijon, France) were surface sterilized for 6 min in 98% sulphuric acid, 5 min in 96% ethanol, an 10 min in 3% calcium hypochlorite, then rinsed in sterile distilled water. They were germinated for 48 h on 0.7% Bactoagar (Difco Laboratories, Detroit) at 25°C in the dark and transplanted into a 75-ml sterilized (autoclaved twice at 120°C) mixture of Terragreen (OilDri-US special, Mettman, Germany) and a neutral clay soil (2:1) for noninoculated plants, or in a 75-ml mixture (2:1) of Terragreen and a soil-based inoculum (spores, roots, and hyphae) of *G. mosseae* Nicol & Gerd., isolate BEG 12, for inoculated plants. Inoculum was prior checked for absence of *S. meliloti* by growing it for 3 weeks with the hypermodulating *M. truncatula* genotype TR122. Control plants received filtered washings (Whatman no. 2; Whatman International, Ltd., Maidstone, England) of *G. mosseae* inoculum to reconstitute associated microflora. Plants were grown under constant conditions: 70% relative humidity, 16-h photoperiod, night and day temperatures of 19 and 22°C, respectively, 360 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (LI-189 LI-COR radiation sensors; LI-Cor, Lincoln, NB, U.S.A.), and received 5 ml of Long Ashton solution without phosphate weekly (Hewitt 1966). Plants were harvested 5 or 10 dai for the J5 genotype and 7 dai after inoculation for the TRV25 mutant. Fresh shoot and root weights were recorded and roots were stored immediately in liquid nitrogen. Root systems of three plants per experiment were digested for 48 h in 10% KOH at room temperature and stained overnight with 0.05% trypan blue in glycerol, to estimate the number of appressoria on roots and evaluate mycorrhiza colonization according to the MYCOCALC program.

In order to determine whether physical contact between the symbionts was necessary for gene expression, the following biological system was developed. Spores of *G. mosseae* were surface sterilized for 15 min in a solution of chloramine T (2%), streptomycin (0.02%), gentamycin (0.01%), and Tween 20 (1 drop 100 μl^{-1}), then germinated for 15 days between sterile membranes of nitrocellulose (Schleicher and Schuell, Dassel, Germany) in autoclaved moist quartz sand. The sandwich of germinated spores was placed over 3-cm-long root systems of 2-day-old *M. truncatula* seedlings held between sterile nitrocellulose membranes in petri dishes (9 cm in diameter) containing the autoclaved Terragreen/soil (2:1) mix. Root systems of control plants were covered with an empty membrane sandwich. Plants were grown in the same conditions as above for 5 (J5 genotype) or 7 (*dmi3* mutant TRV25) days. Spore germination and hyphal growth was verified microscopically on all membranes at plant harvest after staining in a drop of 0.1% methyl blue in 50% lactic acid. Random samples of three seedlings were stained with trypan blue as described above, in order to verify the absence of contact between the symbionts.

In order to compare expression related to mycorrhizal and nodulation interactions, rhizobacteria were introduced into the substrate as a suspension in Long Ashton solution without nitrogen containing 10^6 CFU ml^{-1} of *S. meliloti* strain RCR 2011. Control plants received only Long Ashton solution. Plants were harvested for each treatment 4 dai and immediately stored in liquid nitrogen. Root colonization by the bacteria was evaluated from a sample of five plants after plating serial dilutions of rhizoplane suspensions on King B agar (King et al. 1954), as described by Sanchez and associates (2004).

RNA extraction for SSH library construction.

Noninoculated or *G. mosseae*-inoculated *M. truncatula* roots were extracted according to the method of Franken and

Gnädinger (1994). Frozen roots were ground to a fine powder in liquid nitrogen and transferred to NTES buffer (50 mM Tris HCL, pH 9, 5 mM EDTA, 150 mM NaCl, 5% sodium dodecyl sulfate (SDS), and 1/100 vol of β -mercaptoethanol). Nucleic acids were separated from proteins by three sequential extractions in 1 vol of phenol (pH 4.5-5)/chloroform/isoamyl alcohol (25/24/1) and one in chloroform (1 vol). The RNA in the aqueous phase was purified by precipitation overnight at -20°C in 0.05 vol of 1 M acetic acid and 0.7 vol of 95% ethanol, 4 h at 4°C in 1 vol of 4 M lithium chloride, and overnight at -20°C in 0.1 vol of 3 M sodium acetate and 2.5 vol of 95% ethanol. All RNA solutions were prepared with diethyl pyrocarbonate (DEPC) water (Sigma, Saint Quentin-Fallavier, France). The final RNA pellets were washed with 75% ethanol and were resuspended in 50 μl of DEPC water per 0.5 g of root. RNA in the aqueous phase was treated with DNase for 30 min at 37°C (25 μg of total RNA, 40 U of RNase inhibitor, 3 U of RNase-free DNase, 6 μl of 10 $\times$ buffer, and DEPC water to 60 μl). DNase was removed by phenol/chloroform/isoamyl alcohol (25/24/1), and RNA was precipitated overnight at -20°C (1/10 vol of 3 M sodium acetate, 2.5 vol of 95% ethanol) and resuspended in DEPC water. RNA quantity and quality were estimated by measuring the absorbance at 260 and 280 nm and by 1% denaturing gel electrophoresis.

SSH cDNA-library.

The SSH library was constructed using the PCR Select system according to the protocol provided by the supplier (Clontech Laboratories, Palo Alto, CA, U.S.A.). Briefly, 2 μg of mRNA from 5-day-old *G. mosseae*-inoculated (four appressoria per cm of root) and noninoculated roots were used as template to synthesize two cDNA populations which then were digested with the *RsaI* restriction enzyme. Two different adaptors carrying PCR and cloning sites were ligated to the cDNA from inoculated roots in order to obtain two populations of tester cDNA. No adaptors were added to noninoculated root cDNA (driver cDNA). After verification of the ligation efficiency, each tester cDNA was subtracted by hybridizing it to half of the driver cDNA, followed by a second hybridization of the two subtracted tester cDNA samples. Thereafter, cDNA fragments corresponding to differentially expressed transcripts from the inoculated root cDNA were amplified and cloned into the pGEM-T vector (Promega France, Charbonnière-les-Bains, France).

In order to check subtraction efficiency of DNA samples, expression of the *gapdh* gene of *M. truncatula* (encoding a glyceraldehyde phosphate dehydrogenase) was evaluated using a 1:10 dilution of the subtracted and nonsubtracted cDNA samples as template and *Gapfor* (TGA GGT TGG AGC TGA TTA CG) and *Gaprev* (AGC CTT GGC AGC TCC AGT GC) primers designed on the consensus sequence of the *Mtgap1* cluster MtC00030_GC. 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 of *Taq* polymerase (Qbiogene, Illkirch, France), and 1 $\times$ buffer provided by the supplier of the enzyme. After an initial denaturation of 5' at 95°C , 10, 12, 15, or 17 amplification cycles were performed for 45 s at each temperature step (denaturing, 94°C ; annealing, 55°C ; and elongation, 72°C), with a final extension step of 5 min at 72°C . RT-PCR analysis with specific *Mtgap1* primers gave an amplification product at 15 cycles for the nonsubtracted sample and a weaker signal at 17 cycles for the subtracted sample, showing that the subtraction had been efficient.

Reverse Northern hybridization of cDNA arrays and EST sequencing.

PCR reactions were conducted on ESTs from the SSH library in a 50- μl reaction volume containing 1 μl of 1:100

cDNA clone in water, 0.5 U of *Taq* polymerase (Qbiogene), 125 μ M dNTP, and 0.5 μ M each primer (18.1for GTCACGACGTTGTAAAACG and 18.2rev AGCTATGACCA TGATTACG). Amplifications consisted of an initial denaturation step for 1 min at 95°C; 30 cycles with annealing for 1 min at 56°C and 1 min 30 at 72°C; and a final extension for 5 min at 72°C. PCR products were separated on 1.2% agarose gels, transferred to Hybond-XL (Amersham Bioscience, Orsay, France) by capillary blotting, and fixed under UV light (70,000 J cm⁻²).

cDNA, synthesized from 2.5 μ g of total RNA, was ³²P-labeled by RT-PCR and purified on ProbeQuant G-50 Micro Columns (Amersham Bioscience) before denaturing for 5 min at 95°C. Membranes were prehybridized for 1 h at 60°C and hybridized with probes overnight at 60°C in Church buffer (Church and Gilbert 1984), then washed twice for 5 min in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)/0.1% SDS at room temperature, twice for 20 min in 0.5× SSC/0.1% SDS at 60°C, and twice for 20 min in 0.5× SSC/0.1% SDS at 65°C. Hybridization signals were quantified in a Storm 860 phosphorimager with ImageQuant software (Molecular Dynamics, Amersham Bioscience) and normalized using the *Mtgap1* gene.

ESTs corresponding to genes showing more than 2.5-fold expression in *G. mosseae*-inoculated roots were sequenced using T7 and SP6 primers (MWG-Biotech, Ebersberg, Germany). Sequences of cDNA were compared using blastn and blastx algorithms with sequences in the TIGR *M. truncatula* gene index database to identify similarities at nucleic and amino acids levels.

Semi-quantitative RT-PCR.

Total RNA was isolated using the RNA easy Plant Mini kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. cDNA was prepared from 2 μ g of total RNA added to 1.5 μ g of oligodT₁₅ dNTPs (2.5 mM each) and made up to a final volume of 11.5 μ l with sterile distilled water. RNA was denatured for 5 min at 70°C, placed on ice, and 5 μ l of MMLV 5× reaction buffer, 300 U of MMLV reverse transcriptase, and 80 U of 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. Semi quantitative RT-PCR was performed according to Taylor and Harrier (2003). Gene-specific fragments were amplified by PCR using primers deduced from EST sequences at 95°C for 5 min, 93°C for 45 s, annealing for 60°C for 45 s, 72°C for 1 min, and a final extension at 72°C for 5 min. Amplification products were analyzed by 1.2% agarose gel electrophoresis, stained by ethidium bromide, and quantified in a Storm 860 phosphorimager with ImageQuant software (Molecular Dynamics, Amersham Bioscience).

To further verify that *G. mosseae* had not come into contact with *M. truncatula* when germinated spores were separated from root systems by a nitrocellulose membrane, PCR was performed to detect fungal cDNA in roots separated from the fungus and compared with roots directly inoculated at 5 and 10 dai. Specific primers of the LSU rRNA of *G. mosseae* were used: 5.25 (ATCAACCTTTTGAGCTCG) (van Tuinen et al. 1998) and FLR2 (GTCGTTTAAAGCCATTACGT) (Trouvelot et al. 1999). PCR conditions were 94°C for 5 min; 30 cycles of 92°C for 45 s, annealing for 60°C for 45 s, and elongation at 72°C for 1 min; with a final elongation of 1 min at 72°C. PCR products were observed by 1.4% agarose gel electrophoresis after staining with ethidium bromide.

Transcripts of the *Mtgap1* gene were used to verify equivalent RT to cDNA and equivalent amplification in PCR. Expression levels were checked by semi-quantitative PCR of tran-

scripts on cDNA synthesized from RNA of *M. truncatula* roots inoculated or not with *G. mosseae* or *S. meliloti* as described above. PCR was performed on RT products diluted to 1:40 using the *Gapfor* and *Gaprev* primers and amplification products were quantified as described above.

Statistical analyses.

Data for plant growth were analyzed with SYSTAT software. Data from semi-quantitative PCR evaluations of gene expression in replicate RNA batches of noninoculated and *G. mosseae*- or *S. meliloti*-inoculated roots from three independent inoculation experiments were compared using the Student *t* test.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- Institut National de la Recherche Agronomique (INRA) MYCOCALC program:
www.dijon.inra.fr/bbceipm/Mychintec/mycocalc-prg/download.html
- The Institute for Genomic Research (TIGR) *M. truncatula* gene index:
www.tigr.org/tdb/tgi/ntgi
- Medicago EST navigation system (MENS) website:
medicago.toulouse.inra.fr/Mt/EST/