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# Symbiosis-Related Plant Genes Modulate Molecular Responses in an Arbuscular Mycorrhizal Fungus During Early Root Interactions

Pascale M. A. Seddas,<sup>1</sup> Cecilia M. Arias,<sup>1</sup> Christine Arnould,<sup>1</sup> Diederik van Tuinen,<sup>1</sup> Olivier Godfroy,<sup>1</sup> Hassan Aït Benhassou,<sup>1</sup> Jérôme Gouzy,<sup>2</sup> Dominique Morandi,<sup>1</sup> Fabrice Dessaint,<sup>3</sup> and Vivienne Gianinazzi-Pearson<sup>1</sup>

<sup>1</sup>UMR 1088 INRA/5184 CNRS/Université de Bourgogne, Plante-Microbe-Environnement, INRA-CMSE, BP 86510, 21065 Dijon Cedex, France; <sup>2</sup>UMR INRA/CNRS Laboratoire des Interactions Plantes-Microorganismes, BP 52627, 31326 Castanet-Tolosan Cedex, France; <sup>3</sup>UMR Biologie et Gestion des Adventices, INRA-ENESAD-Université de Bourgogne, Dijon, France

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**To gain further insight into the role of the plant genome in arbuscular mycorrhiza (AM) establishment, we investigated whether symbiosis-related plant genes affect fungal gene expression in germinating spores and at the appressoria stage of root interactions. *Glomus intraradices* genes were identified in expressed sequence tag libraries of mycorrhizal *Medicago truncatula* roots by in silico expression analyses. Transcripts of a subset of genes, with predicted functions in transcription, protein synthesis, primary or secondary metabolism, or of unknown function, were monitored in spores and germinating spores and during interactions with roots of wild-type or mycorrhiza-defective (*Myc*<sup>-</sup>) mutants of *M. truncatula*. Not all the fungal genes were active in quiescent spores but all were expressed when *G. intraradices* spores germinated in wild-type *M. truncatula* root exudates or when appressoria or arbuscules were formed in association with wild-type *M. truncatula* roots. Most of the fungal genes were upregulated or induced at the stage of appressorium development. Inactivation of the *M. truncatula* genes *DMI1*, *DMI2/MtSYM2*, or *DMI3/MtSYM13* was associated with altered fungal gene expression (nonactivation or inhibition), modified appressorium structure, and plant cell wall responses, providing first evidence that cell processes modified by symbiosis-related plant genes impact on root interactions by directly modulating AM fungal activity.**

Arbuscular mycorrhiza (AM) associations, which have probably existed since the Ordovician/Devonian period (Redecker et al. 2000), today represent a ubiquitous symbiosis between roots of the large majority of land plants and fungi belonging to the division Glomeromycota (Krings et al. 2007; Schüßler et al. 2001). Plants rely on AM fungi to facilitate uptake of mineral nutrients from the soil, especially phosphate, and, in return, the fungal partner receives photosynthetically fixed carbon (Smith and Read 1997). The biotrophic interfaces that are

formed between plant and fungal cells must result from coordinated developmental programs in both partners and provide specialized platforms for the exchange not only of metabolites but also of molecular information (Gianinazzi-Pearson 1996; Harrison 1998). An extensive list of plant genes that are predicted to be regulated during AM interactions has become available from high-throughput transcriptome profiling (Feddermann et al. 2008; Frenzel et al. 2005; Gianinazzi-Pearson et al. 2004, 2006; Güimil et al. 2005; Hohnjec et al. 2005; Journet et al. 2002; Küster et al. 2004, 2007; Liu et al. 2003, 2007; Manthey et al. 2004; Massoumou et al. 2007; Siciliano et al. 2007), but comparatively few fungal genes have been identified (Balestrini and Lanfranco 2006; Breuninger and Requena 2004; Ferrol et al. 2004) and little knowledge exists about signaling processes between symbionts in AM, the pathways leading to symbiosis-specific development of AM fungi in root tissues, or mechanisms of subsequent nutrient exchange between them (Balestrini and Lanfranco 2006; Gianinazzi-Pearson et al. 2007; Krajinski and Frenzel 2007; Requena et al. 2007; Séjalon-Delmas et al. 2007).

The life cycle of AM fungi comprises several well-defined developmental stages for which the genetic determinants are still unknown: spore germination, presymbiotic hyphal growth, appressorium formation at the host surface, root penetration, proliferation in internal cortical tissues with differentiation of intracellular arbuscules, and formation of an extraradical mycelium where spores are generated (Harrison 1998; Hause and Fester 2005). Gene expression analyses of these different events is hampered by the fact that AM fungi are obligate biotrophs that cannot be cultured in the absence of root tissues, and by the low abundance of fungal transcripts that can be extracted from colonized root tissues, especially at early time points of mycorrhiza development (Maldonado-Mendoza et al. 2002). In spite of this, there is some evidence for the existence of host plant influences on fungal gene activity both prior to and following root colonization.

Most studies report AM fungal gene activation in intraradical or extraradical mycelium and this relates principally to processes of cell wall synthesis, nutrient metabolism, or membrane transport (Ferrol et al. 2004). For example, two chitin synthase genes of *Gigaspora margarita*, which were not expressed in germinating spores (*GimCHS1* and *GimCHS3*), were found to be induced during mycelium extension in host root tissues (Lanfranco et al. 1999). Isoforms of some genes can vary in

Corresponding author: P. M. A. Seddas; E-mail: pседdas@dijon.inra.fr

Current address for C. M. Arias: UMR 8576, Unité de glycobiologie structurale et fonctionnelle, 59491 Villeneuve d'Ascq, France.

Current address for O. Godfroy: UMR INRA/CNRS Laboratoire des Interactions Plantes-Microorganismes, CNRS-INRA, BP 52627, 31326 Castanet-Tolosan Cedex, France.

their expression profiles. This appears to be the case for plasma-membrane H<sup>+</sup>-ATPase genes in *Glomus mosseae*, where *GmHA1* is activated in intraradical hyphae, *GmHA2* is expressed in extraradical hyphae (Ferrol et al. 2000), *GmPMA1* is down-regulated in symbiotic tissues, and *GmHA5* is highly induced in both mycorrhizal roots and extraradical hyphae (Requena et al. 2003). On the other hand, expression of the phosphate transporter genes *GvPT* and *GiPT* is only detected in extraradical mycelium of *G. versiforme* (Harrison and van Buuren 1995) and *G. intraradices* (Maldonado-Mendoza et al. 2001), whereas a gene ortholog from *G. mosseae* (*GmosPT*) is significantly expressed in both intraradical and extraradical mycelium (Benedetto et al. 2005). Studying nitrogen transfer in symbiotic tissues, Kaldorf and associates (1998) reported nitrate reductase (*NR*) gene expression in arbuscules while Govindarajulu and associates (2005) showed that a glutamine synthase gene of *G. intraradices* is preferentially expressed in extraradical hyphae, whereas a gene associated with arginine breakdown is more highly expressed in intraradical mycelium. Other genes that have been investigated are, for the most part, constitutively expressed at different developmental stages of the fungal life cycle (Ferrol et al. 2004).

Far less is known about the events prior to root penetration which are controlled by fungal genes involved in plant recognition and signal transduction, or those active in the regulation of hyphal growth arrest and cytoplasm retraction that takes place in the absence of a host root. Host root exudates and strigolactone derivatives induce prolific branching of hyphae developing from germinating spores of AM fungi (Akiyama et al. 2005; Buée et al. 2000; Gianinazzi and Gianinazzi-Pearson 1990; Giovannetti et al. 1996), and upregulation of a number of genes associated with increased cell activity has been detected in *Gigaspora rosea* during this presymbiotic response (Tamasloukht et al. 2003). On the other hand, Requena and associates (2002) have suggested that a gene coding a putative hedgehog protein with GTPase activity (*GmGIN1*) of *Glomus mosseae*, which is mainly expressed during spore germination prior to contact with the plant and completely shut down during symbiosis, could be involved in the signaling cascade controlling growth arrest and further programmed cell death of hyphae in the absence of a signal from the host plant. Development of appressoria is the main host-specific morphogenetic event indicating recognition of a host root surface by fungal hyphae (Giovannetti et al. 1994; Nagahashi and Douds 1997); however, studies of cell processes related to this developmental stage are still very much in their infancy. A recent analysis of transcriptome modifications in germinated sporocarps of *G. mosseae*, triggered in synchrony with appressorium formation on a host root, identified 27 upregulated genes coding proteins with functions in signaling, transduction, general cell metabolism, defense or stress responses, or of unknown function (Breuninger and Requena 2004). Because two of the proteins have a potential role in calcium-based signaling pathways, it was suggested that Ca<sup>2+</sup> could be involved as a second messenger in the perception of a plant signal leading to appressorium formation.

Plant mutants defective for AM have provided evidence for the existence of a number of genes that control root interactions with symbiotic fungi in *Medicago truncatula*, *Pisum sativum*, *Lotus japonicus*, *Vicia faba*, *Phaseolus vulgaris*, *Melilotus alba*, and *Lycopersicon esculentum* (Borisov et al. 2004; David-Schwartz et al. 2003; Duc et al. 1989; Kistner et al. 2005; Lum et al. 2002; Morandi et al. 2005; Oldroyd and Downie 2004). In *M. truncatula*, three symbiosis-related (SR) genes have been identified: *DMI1* (coding a putative nuclear channel protein) (Ané et al. 2004; Riely et al. 2007), *DMI2/MtSYM2* (coding a receptor like leucine-rich kinase) (Endre et al. 2002; Stracke et al. 2002), and *DMI3/MtSYM13* (coding a nuclear calcium/cal-

modulin-dependent protein kinase) (Lévy et al. 2004; Mitra et al. 2004; Oldroyd and Downie 2006). Orthologs of these three SR genes are upregulated when calcium responses are elicited in soybean cells by *Gigaspora margarita* (Navazio et al. 2007a). Inactivation of *DMI3/MtSYM13* results in the nonperception of AM fungal signals (Weidmann et al. 2004) and mutation of the pea ortholog *PsSYM9/30* leads to activation of plant defense responses to the symbiotic fungi (Gollotte et al. 1993; Ruiz-Lozano et al. 1999). Furthermore, induction of cytoskeletal endoplasmic reticulum rearrangements in response to appressorium formation is impaired in *M. truncatula* mutated for the genes *DMI2/MtSYM2* or *DMI3/MtSYM13* (Genre et al. 2005). These observations infer that the SR plant genes are involved in fungal recognition and in the implementation of root cell responses essential to mycorrhiza development. However, it cannot be excluded that cell processes modified by SR plant genes may also impact on root interactions by directly modulating AM fungal activity, but this has not so far been investigated.

In order to test this hypothesis and so gain better insight into molecular processes determining compatible interactions essential to AM establishment, we have monitored expression of selected *Glomus intraradices* genes at different stages of the fungal cycle life (quiescent or germinating spores, appressoria, and intraradical mycelium) and determined how they are influenced by inactivation of *M. truncatula* SR genes. Mutation of *DMI1*, *DMI2/MtSYM2*, or *DMI3/MtSYM13* resulted in cell wall reactions to the AM fungus and in differential nonactivation or repression of fungal genes at the stage of spore germination or appressorium formation on the root surface of *M. truncatula* suggesting that, in addition to fungal signal perception, SR plant genes are involved in symbiotic processes modulating AM fungal activity through the production of stimulatory plant factors or the control of inhibitory plant reactions.

## RESULTS

### Selection of *G. intraradices* genes potentially active in mycorrhiza.

Blastn comparisons of partial sequences of 5,051 protein-encoding genes from members of Glomeromycota available in public databases against 3,034 expressed sequence tag (EST) clusters from the MtC *M. truncatula* mycorrhizal root library gave 93 sequences with a score of more than 95% similarity over 100 bp. Fourteen of these sequences were selected on the basis of the predicted function of the corresponding genes (Table 1). Fungal identity was confirmed by polymerase chain reaction (PCR) on genomic DNA from *G. intraradices* spores followed by sequencing, and by the absence of amplification using the corresponding gene primer pair in PCR on cDNA from noninoculated *M. truncatula* roots.

### Appressorium development on roots of wild-type and mutant *M. truncatula* genotypes.

Alkaline phosphatase staining detected metabolically active appressoria in contact with roots of wild-type J5 *M. truncatula* from 5 to 9 days after inoculation (dai) and of the three symbiosis-defective mutants from 7 to 11 dai. Thereon, the number of appressoria per centimeter of root formed during the first day of *G. intraradices*-*M. truncatula* contact (5 dai for the wild-type J5 and 7 dai for the three symbiosis-defective mutants) was not significantly different between the *M. truncatula* genotypes (Table 2). *G. intraradices* penetrated wild-type J5 roots 7 dai and, at 28 dai, the fungus had colonized 44.4 ± 3.2% of the root system of wild-type *M. truncatula* and arbuscules were present in 78.3 ± 5.6% of the mycorrhizal tissues. The shape of appressoria was influenced by the plant geno-

type. Elongated lens-shaped appressoria developed at cell junctions on wild-type J5 (Fig. 1A and B) whereas they had a thicker or ill-defined form on *dmi2/Mtsym2* (Fig. 1G and H) or *dmi3/Mtsym13* (Fig. 1J and K) roots and were frequently surrounded by a layer of translucent material (Fig. 1G, H, and J). Typical fork structure of several appressoria were observed when formed on *dmi3/Mtsym13* roots (Fig. 1K). Appressoria forming on roots of the *dmi1* mutant developed irregular outgrowths (Fig. 1D and E) which did not penetrate the root beyond the epidermis. Plant cell wall modifications were observed below the appressoria. Whereas the epidermal cell walls of wild-type J5 remained thin (Fig. 1C), those of the three symbiosis-defective mutants became thickened (Fig. 1F, I, and L), and stratified in the case of *dmi2/Mtsym2* (Fig. 1I) and *dmi3/Mtsym13* (Fig. 1L), where electron-dense material was frequently deposited at the surface of the fungus.

### Transcript accumulation during mycorrhiza development.

Transcript accumulation of the 14 *G. intraradices* genes was monitored by reverse-transcription (RT)-PCR in quiescent spores, appressoria (5 dai), and intraradical mycelium (28 dai). *G. intraradices* large-subunit (LSU) rRNA was included in transcript analyses to ensure equivalent levels of fungal RNA at the different developmental stages (Fig. 2). Gel analysis of RT-PCR products of genes encoding the  $\alpha$  subunit of translation elongation factor 1 (*TEF*) and the tubulin  $\alpha$  chain (*TUB*) indicated similar transcript levels at each stage of *G. intraradices* development whereas all the other protein-encoding genes presented expression profiles which varied between spores, appressoria, or intraradical mycelium (IRM) (Fig. 2). All the 14 fungal genes were clearly active in IRM and, to different extents, in spores or appressoria (Fig. 2). No transcripts for those coding a 26S proteasome regulatory subunit (*26SREG*), a thio-

**Table 1.** Putative functions of selected genes of *Glomus intraradices*, cluster identity (ID) in MENS, primer sequences, annealing temperature, and amplicon size

| Category of protein-encoding genes   | ID MENS       | PCR primers <sup>x</sup>   |   | Temp (°C) <sup>y</sup> | Size (bp) <sup>z</sup> |
|--|---------------|----------------------------|---|------------------------|------------------------|
|  |               | Name                       | Sequence  |                        |                        |
| Signalling, transcription, protein turnover<br>Translation elongation factor1, $\alpha$ subunit ( <i>TEF</i> ) | MtD00004.2_GC | TEF for<br>TEF rev         | AGCCGAACGTGAACGTGG<br>GCACAATCGGCCTGAGAAGTAC      | 55                     | 247                    |
| Rho/GDP dissociation inhibitor ( <i>RHO</i> )  | MtC91640_GC   | RHO for<br>RHO rev         | GGAATCAGGGTAGATAAAAAC<br>AGTAAATFATTAATCCCAATCC   | 55                     | 214                    |
| 60S ribosomal protein ( <i>60SRIB</i> )  | MtC30534_GC   | 60SRIB for<br>60SRIB rev   | GAATTCGTATCGACTTTGCG<br>GTACGGTATCATCAGATCAC      | 60                     | 376                    |
| Peptidylprolyl isomerase ( <i>PEPISOM</i> )  | MtC00626_GC   | PEPISOM for<br>PEPISOM rev | GATGTTTCATGCCGGTAAAAAG<br>ACTGGATGAACCCAATGTCT    | 55                     | 235                    |
| 26S proteasome subunit ( <i>26S</i> )  | MtC91373_GC   | 26S for<br>26S rev         | CACGTGTTTTGCCAACGC<br>GCTTCTTCTTTCAGTAAC          | 55                     | 183                    |
| 26S proteasome regulatory subunit ( <i>26SREG</i> )  | MtC91656_GC   | 26SREG for<br>26SREG rev   | CCTACTTTTGACCGACGTCA<br>CAGTCTTCTTAACATTGGCT      | 55                     | 231                    |
| Carboxypeptidase precursor ( <i>PRECARB</i> )  | MtC91674_GC   | PRECARB for<br>PRECARB rev | GCAGCAGAAGATACAAAATG<br>CTTATGACCAAGCGTCAG        | 55                     | 129                    |
| Lipid metabolism<br>Stearoyl-CoA desaturase ( <i>DESAT</i> )   | MtC91345_GC   | DESAT for<br>DESAT rev     | TCGTGTTCTGAAAATGAAG<br>GCTTTAGTGGAGTCTTTACC       | 55                     | 269                    |
| Antioxidative metabolism<br>Thioredoxin peroxidase ( <i>THIO</i> )   | MtC91876_GC   | THIO for<br>THIO rev       | GGGAAGCTAAGGCAGTTGT<br>CGCAATATGAGCGTGATGT        | 60                     | 214                    |
| Cu/Zn Superoxide dismutase ( <i>SOD</i> )  | MtD23013_GC   | SOD for<br>SOD rev         | CTGGACCTCATTTTAACCCA<br>CCGATAACACCACAAGCAA       | 55                     | 273                    |
| Cytoskeleton<br>Tubulin $\alpha$ chain ( <i>TUB</i> )  | MtC50940_GC   | TUB for<br>TUB rev         | CGGTGAAGGTATGGAAGAAG<br>GGC CAC CAA GTC ACG AAA A | 60                     | 361                    |
| Unknown function<br>Hypothetical protein (vacuolar import) ( <i>H</i> )  | MtC91226_GC   | H for<br>H rev             | CGCTCCGAACCTTTTATGGAA<br>CCGAGGACTATCCTGAGTT      | 55                     | 345                    |
| Unknown 1 ( <i>U1</i> )  | MtC00279_GC   | U1 for<br>U1 rev           | CGCGTAACCCACTATTTAAGTG<br>CCAACCTTTGCAACTTTA CCG  | 60                     | 285                    |
| Unknown 2 ( <i>U2</i> )  | MtC30486_GC   | U2 for<br>U2 rev           | AGGCACCTCATGAAACGCTG<br>GTAATCTCGTCAAAGATCACG     | 60                     | 521                    |
| Ribosomal RNA<br>Large subunit rRNA ( <i>LSU rRNA</i> )  |               | LR1<br>8.24                | GCATATCAATAAGCGGAGGA<br>CGATCAGAGACCAGACAGGT      | 60                     | 659                    |

<sup>x</sup> PCR = polymerase chain reaction, for = forward, and rev = reverse.

<sup>y</sup> Annealing temperature.

<sup>z</sup> Amplicon size.

**Table 2.** Number of alkaline phosphatase stained appressoria formed on the four *Medicago truncatula* genotypes between 5 and 11 days after inoculation (dai) with *Glomus intraradices*<sup>z</sup>

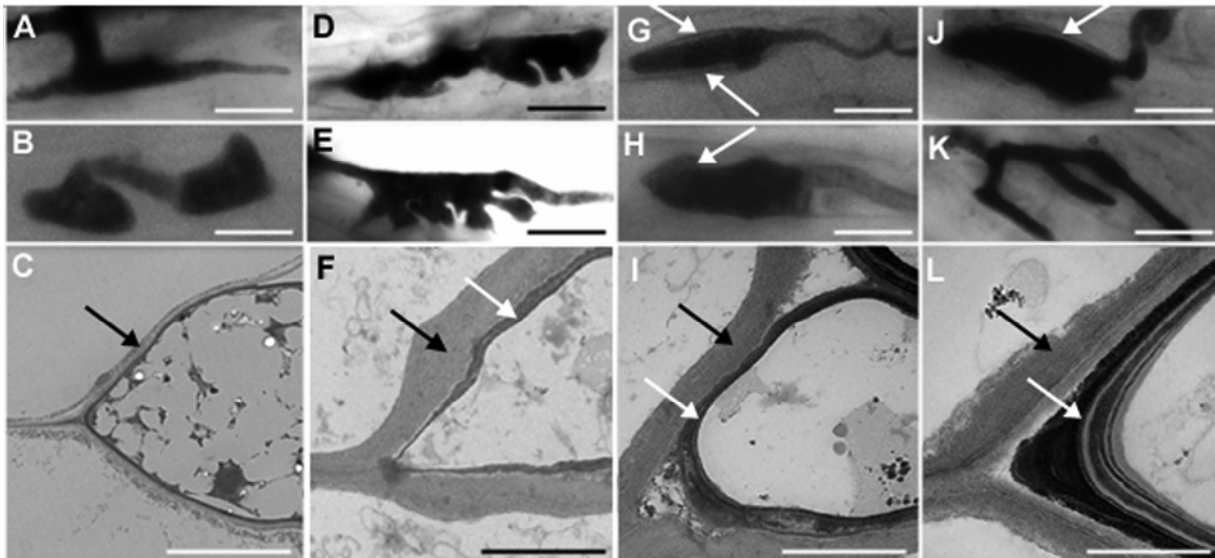
| <i>M. truncatula</i> genotype | 5 dai  | 6 dai  | 7 dai  | 8 dai  | 9 dai  | 10 dai | 11 dai |
|-------------------------------|--------|--------|--------|--------|--------|--------|--------|
| J5                            | 5.14 a | 5.66 a | 5.5 a  | 5.14 a | 4.59 a | n.d.   | n.d.   |
| <i>dmi1</i>                   | 0      | 0      | 5.83 a | 6.26 b | 9.22 d | 7.42 c | 7.38 c |
| <i>dmi2/Mtsym2</i>            | 0      | 0      | 5.61 a | 5.65 a | 6.17 a | 5.92 a | 5.74 a |
| <i>dmi3/Mtsym13</i>           | 0      | 0      | 5.79 a | 6 a    | 6.22 a | 4.5 b  | 3.22 c |

<sup>z</sup> Values in lines followed by the same letter are not significantly different ( $P = 0.005$ ) using one-way analysis of variance and Tukey's pairwise comparisons test with PAST (homogeneity of variance was tested with Levene's test); n.d. = not detected.

redoxin peroxidase (*THIO*), or two unknown proteins (*U1* and *U2*) were detected in spores (Fig. 2), whereas they were induced in appressoria and IRM. Twelve genes were upregulated in intraradical mycelium compared with spores. Expression of *26S*, *26SREG*, *PRECARB*, *U1*, and *U2* showed marked increases in intraradical mycelium compared with appressoria (Fig. 2).

#### Effect of host plant genotype on fungal gene expression.

*G. intraradices* gene expression was analyzed by real-time RT-PCR in quiescent spores, in germinating spores elicited by root exudates, and at the appressorium stage in interactions with roots of wild-type J5 *M. truncatula* and the symbiosis-defective mutants *dmi1*, *dmi2/Mtsym2*, or *dmi3/Mtsym13*. Com-

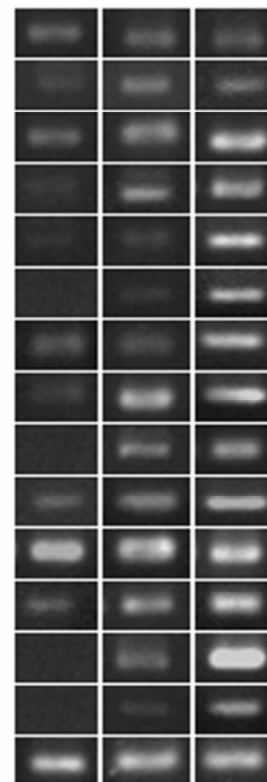


**Fig. 1.** Development of appressoria of *Glomus intraradices* on roots 7 days after inoculation of **A, B, and C**, wild-type J5; **D, E, and F**, *dmi1*; **G, H, and I**, *dmi2/Mtsym2*; and **J, K, and L**, *dmi3/Mtsym13* genotypes of *Medicago truncatula*. **A, B, D, E, G, H, J, and K** present appressoria stained for alkaline phosphatase activity; arrows indicate translucent material surrounding appressoria. Bar = 10  $\mu$ m. **C, F, I, and L** are micrographs of sections through appressoria developing at cell junctions on the root surface; arrows indicate wall thickenings (black) and electron dense deposits (white). Bar = 1  $\mu$ m.

### Protein-encoding genes

Transcription elongation factor1, alpha subunit (TEF)  
 Rho/GDP dissociation inhibitor (RHO)  
 60S ribosomal protein (60SRIB)  
 Peptidylprolyl isomerase (PEPISOM)  
 26S proteasome subunit (26S)  
 26S proteasome regulatory subunit (26SREG)  
 Carboxypeptidase precursor (PRECARB)  
 Stearoyl-CoA desaturase (DESAT)  
 Thioredoxin peroxidase (THIO)  
 Cu/Zn superoxide dismutase (SOD)  
 Tubulin alpha chain (TUB)  
 Hypothetical protein (vacuolar import domain) (H)  
 Unknown 1 (U1)  
 Unknown 2 (U2)  
 Large subunit rRNA (LSU rRNA)

Spores Appr IRM



**Fig. 2.** Transcript accumulation of *Glomus intraradices* genes in spores, appressoria (Appr), and intraradical mycelium (IRM) when fungus developed in contact with J5 roots. Reverse-transcription polymerase chain reaction (RT-PCR) was performed on total RNA with specific primers for each gene and with 26 PCR cycles. PCR products separated by gel electrophoresis were stained with ethyl bromide.

parisons were made at 7 dai for the mutants and at 5 dai on roots of the genotype J5 after first appressoria formation and before root penetration (described above). The same amount of RNA and number of SMART cycles for cDNA synthesis were used for root extracts of the four inoculated plant genotypes and for extracts from germinated spores elicited by root exudates. Gene expression profiles observed by gel analysis of RT-PCR products were confirmed by real-time RT-PCR in quiescent spores and in appressoria developing at the root surface of wild-type J5 *M. truncatula* using the *TEF* gene as a constitutive reference gene, and all data for transcript levels of the *G. intraradices* candidate genes were normalized against values for the *TEF* gene.

### Effect of root exudates on gene expression during spore germination.

Transcripts of most of the studied genes were detected in quiescent spores of *G. intraradices*, except those corresponding to *26SREG*, *THIO*, *U1*, and *U2* (Table 3). *26SREG* and *U1* were induced during germination of spores in water; *PEPISOM* and *SOD* were upregulated; *THIO* and *U2* remained inactive; *RHO*, *26S*, *PRECARB*, *DESAT*, and *H* were expressed at similar levels; and *60S* was downregulated compared with quiescent spores (Table 3). All fungal genes were expressed in spores germinating in root exudates of wild-type J5 *M. truncatula* but only the genes *RHO*, *60S*, *THIO*, *U1*, and *U2* were elicited compared with water controls (Table 3). When spores germinated in root exudates from the mycorrhiza-defective mutants, a number of fungal genes were downregulated or not expressed compared with wild-type J5 root exudates or water controls. Fungal genes were mostly affected by mutation of the SR plant gene *DMI2/MtSYM2* (eight) and, to a lesser extent, by inactivation of *DMI1* (six) or *DMI3/MtSYM13* (five) (Table 3). In the presence of *dmi1* mutant root exudates, the genes *RHO*, *60S*, *PEPISOM*, and *DESAT* were significantly downregulated, *U1* and *U2* were not expressed, and five genes (*26S*, *PRECARB*, *THIO*, *SOD*, and *H*) were expressed to a level similar to that in wild-type J5 root exudates. In contrast, five genes were not expressed in spores germinating in *dmi2/Mtsym2* root exudates (*26SREG*, *DESAT*, *THIO*, *U1*, and *U2*), three were significantly downregulated (*RHO*, *60S*, and *SOD*), and only four had transcript levels comparable with those germinating in J5 root exudates (*PEPISOM*, *26S*, *PRECARB*, and *H*). The genes *THIO*, *U1*, and *U2* were likewise not expressed in spores germinating in *dmi3/Mtsym13* root exudates, whereas no significant difference was observed for five other genes (*60S*, *26S*, *DESAT*, *SOD*, and *H*) compared with J5 root exudate-incubated spores, except for *PEPISOM*

and *PRECARB*, which were significantly upregulated whereas *RHO* and *26SREG* were downregulated.

### Gene expression associated with appressorium development.

*G. intraradices* transcript levels were similar for *26S* and *THIO* genes in appressoria developing on roots of all four genotypes of *M. truncatula* (Table 4). Fungal genes were mostly downregulated or not expressed in contact with roots of the mutants *dmi2/Mtsym2* (seven) or *dmi3/Mtsym13* (six) and, to a lesser extent, with *dmi1* (five) (Table 4). Mutation of the *DMI1* gene of *M. truncatula* decreased or inhibited expression of the *60S*, *PEPISOM*, *DESAT*, *U1*, and *U2* genes in appressoria in much the same way as corresponding root exudates. A more pronounced effect on *G. intraradices* gene expression was observed with appressorium formation on *dmi2/Mtsym2* roots: all the *G. intraradices* genes that were downregulated or not expressed in root exudates of the mutant, compared with wild-type J5 exudate, behaved similarly, except for *THIO*, which is activated and expressed at the same level as in appressoria formed on wild-type J5 such as *SOD*, and *PRECARB*, which is downregulated compared with wild-type J5 roots. When appressoria formed on *dmi3/Mtsym13* roots, the *U1* and *U2* genes were again not expressed; however, no effect on the *60S*, *PEPISOM*, *26S*, *26SREG*, *THIO*, and *SOD* genes was observed whereas *RHO*, *PRECARB*, *DESAT*, and *H* were down-

**Table 4.** Transcripts of *Glomus intraradices* genes (relative to translation elongation factor 1 [*TEF*]) quantified by real-time polymerase chain reaction during appressorium formation on roots of wild-type (J5) and mycorrhiza-defective (*Myc*<sup>-</sup>) mutant genotypes (*dmi1*, *dmi2/Mtsym2*, and *dmi3/Mtsym13*) of *Medicago truncatula*<sup>z</sup>

| Gene           | J5      | <i>dmi1</i> | <i>dmi2/Mtsym2</i> | <i>dmi3/Mtsym13</i> |
|----------------|---------|-------------|--------------------|---------------------|
| <i>RHO</i>     | 6.21 a  | 6.00 a      | 3.42 b             | 3.85 b              |
| <i>60S</i>     | 7.66 a  | 6.14 b      | 6.00 b             | 8.06 a              |
| <i>PEPISOM</i> | 9.34 a  | 2.68 b      | 8.22 a             | 8.26 a              |
| <i>26S</i>     | 5.91 a  | 5.69 a      | 4.99 a             | 6.11 a              |
| <i>26SREG</i>  | 4.23 a  | 4.86 a      | 0 b                | 4.31 a              |
| <i>PRECARB</i> | 10.04 a | 9.97 a      | 7.75 b             | 8.19 c              |
| <i>DESAT</i>   | 9.54 a  | 0.50 b      | 0 b                | 3.00 c              |
| <i>THIO</i>    | 1.42 a  | 2.06 a      | 1.95 a             | 1.12 a              |
| <i>SOD</i>     | 3.60 ab | 4.00 a      | 2.13 b             | 3.00 ab             |
| <i>H</i>       | 3.62 a  | 3.36 a      | 4.43 a             | 1.96 b              |
| <i>U1</i>      | 10.31 a | 0 b         | 0 b                | 0 b                 |
| <i>U2</i>      | 4.86 a  | 0 b         | 0 b                | 0 b                 |

<sup>z</sup> Values in lines followed by the same letter are not significantly different ( $P = 0.005$ ) using one-way analysis of variance and Tukey's pairwise comparisons test with PAST (homogeneity of variance was tested with Levene's test).

**Table 3.** Transcripts of *Glomus intraradices* genes (relative to translation elongation factor 1 [*TEF*]) quantified by real-time polymerase chain reaction in quiescent spores and spores germinated in water or in root exudates from wild-type J5 and mycorrhiza-defective (*Myc*<sup>-</sup>) mutant genotypes (*dmi1*, *dmi2/Mtsym2*, and *dmi3/Mtsym13*) of *Medicago truncatula*<sup>z</sup>

| Gene  | Quiescent | Water   | J5      | <i>dmi1</i> | <i>dmi2/Mtsym2</i> | <i>dmi3/Mtsym13</i> |
|---|-----------|---------|---------|-------------|--------------------|---------------------|
| <i>RHO</i> (Rho/GDP dissociation inhibitor)       | 5.39 a    | 5.29 a  | 5.78 b  | 5.39 a      | 4.73 c             | 4.65 c              |
| <i>60S</i>  | 8.95 a    | 5.79 b  | 8.81 a  | 7.25 c      | 6.69 bc            | 8.64 a              |
| <i>PEPISOM</i> (peptidylprolyl isomerase)         | 1.83 a    | 5.64 b  | 6.60 b  | 2.89 a      | 5.53 b             | 9.57 c              |
| <i>26S</i> (26S proteasome subunit)               | 2.66 a    | 2.12 a  | 2.43 a  | 2.41 a      | 2.53 a             | 3.19 a              |
| <i>26SREG</i> (26S proteasome regulatory subunit) | 0 a       | 2.12 b  | 2.51 b  | 3.71 c      | 0 a                | 0.32 a              |
| <i>PRECARB</i> (carboxypeptidase precursor)       | 5.34 a    | 5.39 a  | 5.47 a  | 5.46 a      | 5.21 a             | 6.50 b              |
| <i>DESAT</i> (stearoyl-CoA desaturase)            | 4.59 a    | 3.30 ac | 3.40 ac | 0.51 b      | 0 b                | 2.62 c              |
| <i>THIO</i> (thioredoxin peroxidase)              | 0 a       | 0 a     | 1.61 b  | 2.21 b      | 0 a                | 0 a                 |
| <i>SOD</i> (Cu/Zn superoxide dismutase)           | 2.19 a    | 9.25 b  | 8.53 b  | 8.96 b      | 7.05 c             | 8.60 b              |
| <i>H</i> (hypothetical protein)                   | 1.29 a    | 1.26 a  | 1.26 a  | 1.32 a      | 1.34 a             | 1.36 a              |
| <i>U1</i> (Unknown 1)                             | 0 a       | 1.07 b  | 2.84 c  | 0 a         | 0 a                | 0 a                 |
| <i>U2</i> (Unknown 2)                             | 0 a       | 0 a     | 2.38 b  | 0 a         | 0 a                | 0 a                 |

<sup>z</sup> Values in lines followed by the same letter are not significantly different ( $P = 0.005$ ) using one-way analysis of variance and Tukey's pairwise comparisons test with PAST (homogeneity of variance was tested with Levene's test).

regulated compared with appressoria formed on wild-type J5, with root contact.

## DISCUSSION

Cell programs of AM fungi must undergo continual adaptation during the different developmental stages leading to the formation and functioning of the mycorrhizal symbiosis. Previous studies have mainly focused on modifications in fungal gene expression during colonization of host tissues (Ferrol et al. 2004), and very few have targeted early gene responses to root exudates (Tamasloukht et al. 2003) or gene activation in synchrony with appressorium formation on host roots (Breuninger and Requena 2004). Here, we provide evidence for a role of SR plant genes in the early activation of AM fungal genes putatively implicated in cellular signaling (*RHO*), protein turnover (*60SRIB*, *PEPISOM*, *26S*, *26SREG*, and *PRECARB*), lipid metabolism (*DESAT*), or antioxidative processes (*THIO* and *SOD*), or of unknown function (*H*, *U1*, and *U2*). Most of the modifications in gene responses of *G. intraradices* to *M. truncatula* roots required cell contact (appressorium stage) but root exudates were sufficient to modulate the molecular response of certain fungal genes (activation or inhibition). The response of AM fungi to host-exuded factors during precolonization stages of interactions is a well-described phenomenon (Buée et al. 2000; Gianinazzi and Gianinazzi-Pearson 1990; Giovannetti et al. 1996; Sbrana and Giovannetti 2005; Tamasloukht et al. 2003), although the chemical nature of host compounds acting as recognition and growth-enhancing signals for the fungal symbionts is still poorly defined. Both water-soluble and volatile compounds can be effectors (Buée et al. 2000; Gemma and Koske 1988; Nagahashi and Douds 1999), among which flavonoids and strigolactones have been proposed as candidates (Akiyama et al. 2005; Vierheilg and Piché 2002).

Root exudates of wild-type *M. truncatula* stimulated expression of five genes in germinating spores of *G. intraradices* compared with water. All the genes, except *RHO* and *SOD*, were upregulated when appressoria of *G. intraradices* differentiated in contact with root cells of wild-type *M. truncatula* and all were active in intraradical mycelium. In contrast, most of the *G. intraradices* genes were consistently downregulated or not activated by exudates from or contact with roots of one or other of the symbiosis-defective (mycorrhiza-defective [Myc<sup>-</sup>]) *M. truncatula* mutants. Fungal gene expression was most affected by mutation of the SR *DMI2/MtSYM2* and *DMI3/MtSYM13* genes and, to a lesser extent, by inactivation of *DMII*. This implies that the control of SR plant genes over the complex dialogue needed for AM establishment involves modulation of fungal activity in the early stages of interactions even prior to cell contact between the partners. On the plant side, recent work has shown that the mutation of only one plant SR gene can lead to the modulation of several hundred others at the appressorium stage of fungal development. *Medicago* GeneChip (Benedito et al. 2008) transcriptome profiling of *G. intraradices*-inoculated versus noninoculated *M. truncatula* wild-type and symbiosis-defective mutant roots at appressoria formation revealed that, although few encoding transcription factor are modulated in wild-type (7 factors) and *dmi2/MtSYM2* (5 factors) roots, more than 25 and 50 are downregulated in *dmi1* and *dmi3/MtSYM13* mutant roots, respectively (Seddas et al. 2009). Likewise, more genes that are implicated in cellular signalization are modulated in *dmi1* and *dmi3/MtSYM13* mutant roots than in wild-type and *dmi2/MtSYM2* roots. All together, these results underline the very complex molecular mechanisms that must be triggered in the mycorrhizal partners when an AM fungus comes into contact with roots. We can also hypothesize that the delay of 2 days between the

formation of appressoria on wild-type roots or on roots of the three symbiosis-defective mutants could be due to a delay of synthesis of a signal molecule in the mutants or to the fact that *G. intraradices* has to activate some specific genes to overcome the inhibitory molecules produced by these mutants.

Some of the *G. intraradices* genes were similarly affected by the *M. truncatula* mutants during both spore germination and root contact. This was the case for the two genes *U1* and *U2* of unknown function which were consistently inactive or downregulated in the presence of all three *M. truncatula* mutants compared with their expression levels in interactions with the wild-type plant genotype. The *M. truncatula* mutants differentially affected the activity of other fungal genes during both spore germination and root contact: *dmi2/MtSYM2* and *dmi3/MtSYM13* downregulated *RHO* whereas *dmi1* and *dmi2/MtSYM2* downregulated *60S* and *DESAT*, *dmi1/MtSYM1* reduced *PEPISOM* expression, and *dmi2/MtSYM2* didn't activate *26SREG* expression. The strong downregulation of the stearoyl-CoA desaturase (*DESAT*) gene of *G. intraradices* suggests modifications in fungal lipid metabolism, and this may also be an element contributing to alterations in fungal development in the presence of the mutant roots. Known fungal fatty acid desaturases are all endoplasmic reticulum (ER) membrane-bound enzymes which have their active site on the ER's cytoplasmic face (Wongwathanarat et al. 1999). The transcription of such a gene, for example in yeast, is influenced by environmental factors such as temperature and exogenous fatty acids (McDonough et al. 1992) and modifications in these enzymes can alter the fatty acid composition of a fungus (Kawashima et al. 1997). *PEPISOM*, a peptidylprolyl isomerase, plays a role in cellular regulation as a chaperon protein that activates or inhibits regulatory proteins by catalyzing proline isomerization (Miele et al. 2003), while the *26SREG*-regulated proteasome is a protease complex that is responsible for selective degradation of abnormal proteins and naturally short-lived proteins related to metabolic regulation and cell-cycle progression (Murray 1995). The weaker expression of the *RHO* gene, encoding a putative Rho/GDP dissociation inhibitor, could reflect modifications in fungal cell signal events during appressorium development on mutant roots. GTP-binding proteins are active only in the GTP-bound state. GDP-association inhibitors affect the interconversion of GTP (active state) to GDP (inactive state) on Rho/GTPase proteins. Regulation of Rho/GTPase activity, which can be controlled by extracellular signals, is integral to many basic cellular functions such as cell cycle, movement and migration, metabolism, survival, proliferation, and differentiation (Groisman et al. 2002). Furthermore, inhibition of Rho/GTPase activity can result in blocking of calcium mobilization (Groisman et al. 2002) which may be relevant to altered signaling phenomena resulting from inactivation of the SR genes of *M. truncatula*. In contrast, the *PRECARB* gene of *G. intraradices* was only downregulated by root contact with *dmi2/MtSYM2* or *dmi3/MtSYM13*, and the *DESAT* and *H* genes by *dmi3/MtSYM13*.

Genes encoding the oxidative stress response-related proteins thioredoxin peroxidase (*THIO*) and Cu/Zn superoxide dismutase (*SOD*) have been previously reported to be active in AM fungi (Brechenmacher et al. 2004; Lanfranco et al. 2005). The *THIO* gene of *G. intraradices* was induced by root exudates of wild-type J5 and *dmi1 M. truncatula*, compared with spore germinated in water, but this induction was generalized to all the plant genotypes after root contact. In contrast, *SOD* expression was strongly enhanced with spore germination in water and whatever the plant genotype, although transcript levels were lower after root contact, especially in interactions with *dmi2/MtSYM2* roots. These observations suggest that Myc<sup>-</sup> mutant roots of *M. truncatula* do not specifically induce a cel-

lular mechanism against oxidative stress in *G. intraradices*, which could eventually protect against incompatible responses preventing root colonization. The *SOD* gene was also found to be expressed at high levels in germinating spores of *Gigaspora margarita* (Lanfranco et al. 2000) and, as for *Glomus intraradices*, during root colonization (Lanfranco et al. 2005). Recently, Seddas and associates (2008) localized *SOD* expression in vesicles but not in arbuscules of *G. intraradices*-colonizing wild-type *M. truncatula* roots.

Whether inactivation of SR genes in *M. truncatula* modifies fungal responses through the lack of a plant signal or secretion of plant inhibitory molecules remains to be elucidated. In relation to the function of SR gene products in the signal transduction pathway after fungal perception, the fact that mutation of *DMI1*, *DMI2/MtSYM2*, or *DMI3/MtSYM13* results in nonactivation of some AM fungal genes should reflect a role of  $Ca^{2+}$  in such recognition events during early steps of symbiosis establishment. In this context, Navazio and associates (2007a and b) have recently shown that soybean cells perceive *G. intraradices* or *Gigaspora margarita* through a  $Ca^{2+}$ -mediated signaling response pathway which is not active in nonhost *Arabidopsis thaliana*. More recently, Kosuta and associates (2008) demonstrated that diffusible AM fungal factors activate a rapid calcium response in soybean cell cultures or *M. truncatula* root hair cells before direct fungal contact. Calcium oscillations, which are only induced by branched hyphae in root hair cells, are likely to prime host cells for fungal colonization. The nature of these inductive AM fungal signals (myc factors) is unknown but their perception is dependent on SR plant genes and altered in plant mutants where the fungus is unable to gain entry to epidermal cells (Kosuta et al. 2008; Weidmann et al. 2004). The mutation of SR plant genes could lead to the nonrecognition of the AM fungus through interference with signal exchange processes essential to symbiosis establishment and, subsequently, to the upregulation of plant defense as has been suggested for *Rhizobium* spp. (Niehaus et al. 1998).

Weak or transient plant defense responses during the early stages of AM interactions have been reported in different fungus-plant combinations (Garcia-Garrido and Ocampo 2002; Gianinazzi-Pearson et al. 1996). This phenomenon may result from the low capacity of a symbiotic fungus to trigger defense mechanisms or its ability to suppress or induce suppression of the plant defense system (Garcia-Garrido and Ocampo 2002; Gollotte et al. 1993). Appressoria of *Glomus intraradices* were metabolically active but their shape differed depending on the genotype of *M. truncatula* and they were surrounded by translucent material in the case of *dmi2/Mtsym2* and *dmi3/Mtsym13* mutants. This may correspond to the cell wall thickenings observed beneath appressoria in the symbiosis-defective mutant roots which recalls previous observations where appressoria of *G. mosseae* induced abnormal root cell wall reactions in the *dmi2/Mtsym2* mutant (Calantzis et al. 2001). Incompatible responses to *G. mosseae*, characterized by deposition of wall defense molecules and upregulation of defense-related genes, have also been described in *Myc*<sup>-</sup> pea plants mutated for the *PsSYM9/psSYM30* gene ortholog of *DMI3/MtSYM13* (Gollotte et al. 1993, Ruiz-Lozano et al. 1999). Likewise, another SR plant gene, *LJSYM4*, is necessary in *Lotus japonicus* for correct host cytoskeleton reorganization in the presence of an AM fungus and avoidance of a hypersensitive-like reaction (Genre and Bonfante 2002). Suppression of plant defense responses has been proposed by Niehaus and associates (1993) for symbiotic *Rhizobium* spp.–legume interactions following observations that they can be induced in alfalfa by an asymbiotic *Sinorhizobium meliloti* mutant. Gollotte and associates (1993) have already hypothesized that, in analogy to plant–pathogen interactions, perception of an AM fungal elicitor by host roots

could lead to activation of symbiosis genes, the products of which could interact or interfere with the expression of defense genes. Evidence that *M. truncatula* perceives AM fungal signals and that inactivation of SR genes interferes with this recognition process does exist (Genre et al. 2005; Weidmann et al. 2004). If SR gene activity is made redundant through mutation, then plant responses could be modified so that defense mechanisms are no longer suppressed. In this context, the lack of upregulation of antioxidative stress mechanisms involving *SOD* or *THIO* in *G. intraradices* when the SR plant genes are inactivated would contribute to the inability of the fungus to cope with defense responses in the plant mutants.

In conclusion, the cell program of an AM fungus must undergo reorganization in order for the symbiont to accomplish the developmental changes leading to mycorrhiza formation. Flavonoids and strigolactones have been proposed as plant signals that promote presymbiotic AM fungal development but those essential to mycorrhiza establishment still have to be identified, as well as the fungal receptors involved. Conversely, fungal signals (Myc factors) induce gene responses in host roots but the plant receptors are unknown. The present observations on *G. intraradices* gene expression during interactions with wild-type and *Myc*<sup>-</sup> genotypes of *M. truncatula* provide a first indication that SR plant genes regulate AM fungal activity through stimulatory pathways or control of inhibitory factors. The inactivation of SR plant genes may modify fungal signaling events which could interfere with plant perception of the fungal symbiont and thereby impact on its morphological transition from appressorium differentiation to the biotrophic phase of root colonization. A future challenge for research will be to identify the secretome of AM fungi and determine how it is regulated by the SR constituents of the plant genome, because certain secreted fungal proteins could be effectors for processes essential to host–fungus interactions, as recently suggested for the obligate biotrophic pathogen *Uromyces fabae* (Kemen et al. 2005).

## MATERIALS AND METHODS

### Biological material.

Seed of *M. truncatula* Gaertn. cv. Jemalong, line J5, as well as corresponding *dmi2/Mtsym2* (TR25) and *dmi3/Mtsym13* (TRV25) mutants, were provided by G. Duc, URLEG, INRA-Dijon, France, and those of the mutant *dmi1* (Y6) by C. Gough, INRA-Toulouse, France. Seedlings of *M. truncatula* were inoculated or not with *G. intraradices* Smith & Schenck, isolate BEG 141, and grown as described by Weidmann and associates (2004). Briefly, seed were surface sterilized and germinated on 0.7% (wt/vol) Bactoagar (Difco Laboratories, Detroit) for 72 h at 25°C in the dark. Seedlings were used for root exudate production or transplanted individually into a 75-ml sterilized (autoclaved once at 120°C during 7 h) mix of Terragreen (OilDri-US special, Mettman, Germany) and a neutral  $\gamma$ -irradiated clay soil (2:1, vol/vol) for uninoculated plants, or into a 75-ml mix (2:1, vol/vol) of Terragreen and a soil-based inoculum of *G. intraradices* BEG 141, for inoculated plants. Plants were grown under constant conditions (420  $\mu\text{E m}^{-2} \text{s}^{-1}$ ; 24 and 19°C, 16 and 8 h, day and night, respectively; 70% humidity) and received 5 ml of Long Ashton solution without phosphate and a double quantity of nitrate twice a week (Hewitt 1966). Plants were harvested 5 to 11 and 28 dai for the J5 genotype and 5 to 11 dai for the three mutants; roots were washed and immediately stored in liquid nitrogen or stained to estimate the number of metabolically active appressoria on roots or to evaluate mycorrhiza colonization. Extraradical mycelium was manually eliminated under a binocular microscope from roots of J5 plants harvested 28 dai. Appressoria number and



shape were monitored between 5 to 11 dai by digesting root systems from three plants in cellulase and pectinase then staining for alkaline phosphatase activity as described by Tisserant and associates (1993). Mycorrhiza colonization was estimated using the MYCOCALC program after KOH digestion and trypan blue staining according to Weidman and associates (2004).

Root exudates were collected by placing five germinated seedlings of *M. truncatula* in 3 ml of sterile water in a six-well plate (Falcon Multiwell, BD Sciences, San Jose, CA, U.S.A.) under constant conditions ( $420 \mu\text{E m}^{-2} \text{s}^{-1}$ ; 24 and 19°C, 16 and 8 h, day and night, respectively) for 7 days. After elimination of all plant residues, exudates were directly used with spores. Spores of *G. intraradices* BEG 141 were collected from a 6-month-old leek pot culture. Roots were washed under running tap water and lacerated in sterile water using a blender, three times during 1 min at maximum power. The resulting suspension was sieved ( $63 \mu\text{m}$ ) and spores were collected; surface-disinfected during 10 min using 2% chloramine T, 0.02% streptomycin, 0.02% gentamycin, and 20  $\mu\text{l}$  of Tween20; then rinsed twice with sterile water. After eliminating the seedlings, 2,000 disinfected spores were introduced into the root exudates in each well and incubated for 7 days at 25°C in 2% CO<sub>2</sub>. Controls were performed by replacing exudates by 3 ml of sterile water. Germinated spores were collected by centrifugation and immediately used for RNA extraction.

### Electron microscopy.

Pieces of roots samples (2 to 3 mm) 5 dai (wild-type) or 7 dai (three symbiosis-defective mutants) were fixed in 2% (vol/vol) glutaraldehyde diluted in 0.2 M cacodylate buffer, pH 7.2, and postfixed for 1 h at 4°C with 1% OsO<sub>4</sub>. After gradient series of dehydration, samples were embedded in epoxy resin. Ultrathin (90-nm) sections were used for localizing 1-4, 1-6 polysaccharides with periodic acid and thiocarbohydrazide-silver proteinate PATAG reaction (Gollotte et al. 1993). Ultrathin sections were double contrast with uranyl acetate and lead citrate for normal ultrastructural observations. All ultrathin sections were observed using an Hitachi (Tokyo) electron transmission microscope 7500 at 80 Kv (INRA-CMSE, Centre de Microscopie, Dijon, France).

### Expression analyses.

Protein-encoding genes from members of division Glomeromycota were selected from the GenBank public database. A Blastn comparison was performed against EST clusters from *M. truncatula* mycorrhizal roots (Journet et al. 2002) (MENS); 93 sequences with a score of more than 95% similarity over 100 bp were selected as potentially fungal genes, and we selected 14 among them for our study because of their potential function and because they were already active at different levels at the appressoria stage on wild-type roots.

Fungal identity of selected genes was confirmed by PCR on genomic DNA, cloning into the PCR-TOPO vector (Takara Bio, Shiga, Japan) as described by the manufacturer, and sequencing (MWG Biotech, Ebersberg, Germany). Genomic DNA was extracted from 1,000 spores of *G. intraradices* according to the modified protocol of Zézé and associates (1994) described by Gollotte and associates (2006).

DNA-free total RNA was extracted from 2,000 *G. intraradices* quiescent or germinating spores, or from 100 mg of *G. intraradices*-inoculated or noninoculated roots of wild-type J5 *M. truncatula* (5 dai) and symbiosis-defective mutants (7 dai), using the SV total RNA Isolation kit (Promega Corp., Madison, WI, U.S.A.), according to the manufacturer's instructions. RNA was quantified using the Qubit (Invitrogen, Carlsbad, CA, U.S.A.) system. Because of the low abundance of fungal transcripts in spores and inoculated roots, cDNA was prepared

from 25 ng (quiescent spores) or 200 ng (germinating spores or plant roots) of total RNA using SMART technology (Takara Bio) in a final volume of 50  $\mu\text{l}$  according to the manufacturer's instructions. long-distance PCR amplification (Takara Bio) was carried out using 10  $\mu\text{l}$  of SMART-cDNA and 18 PCR amplification cycles to obtain DNA in the exponential phase of amplification (Endege et al. 1999; Vernon et al. 2000).

Total RNA was extracted from mycorrhizal (28 dai) and age-equivalent nonmycorrhizal roots of *M. truncatula* J5 according to the method of Franken and Gnädinger (1994), modified as described by Weidmann and associates (2004). RNA quantity and quality were estimated by absorbance at 260 and 280 nm and by electrophoresis in 1% (wt/vol) denaturing agarose gel. cDNA was prepared from 1  $\mu\text{g}$  of total DNaseI-treated (Promega Corp.) RNA using 1.5  $\mu\text{g}$  of oligodT<sub>15</sub>, 300 U of MMLV reverse transcriptase (EC 2.7.7.49), and 80 U of RNase inhibitor for 15 min at 25°C followed by 1 h at 42°C and 2 min at 72°C, as described by Weidmann and associates (2004).

Fungal transcript accumulation was monitored at different stages of *G. intraradices* development (quiescent spores, appressoria, and intraradicular mycelium) by RT-PCR. Fungal gene-specific fragments were amplified using primer pairs deduced from EST sequences (Table 1) or using LR1/8.24 (van Tuinen et al. 1998) for LSU rRNA. Amplification was performed in a Biometra thermocycler (Göttingen, Germany) at 94°C for 1 min, followed by 26 cycles at 94°C for 45 s, the appropriate primer annealing temperature for 45 s, 72°C for 45 s, and a final extension at 72°C for 1 min. Amplification products were analyzed by 2% (wt/vol) agarose gel electrophoresis in 0.1 Tris-acetate-EDTA (4 mM Tris, 0.1 mM EDTA, 2 mM acetic acid) (Fluka, St Gallen, Switzerland), stained with ethidium bromide for 10 min and observed using GelDoc EQ apparatus and the QuantityOne-4.5.1 computer program to determine the ratio of *TEF* to *LSU rRNA* transcripts (Bio-Rad Inc., Hercules, CA, U.S.A.).

Absolute quantitative real-time RT-PCR was performed for quiescent or germinating spores and at the appressorium stage of *G. intraradices* development on roots of wild-type J5 or symbiosis-defective *M. truncatula* mutants, according to Güimil and associates (2005). In preliminary experiments, the *LSU rRNA*, *TEF*, and *DESAT* genes were selected to compare expression in quiescent spores using either oligodT<sub>15</sub> or SMART cDNA fragments. Because statistically comparable expression levels were obtained in both cases, SMART cDNA was used in quantitative real-time RT-PCR to overcome the low abundance of fungal transcripts detected at the appressorium stage. Fungal gene-specific cDNA fragments were amplified by PCR using QuantiTect SYBR GreenPCR mix (Qiagen, Hilden, Germany), the same sets of primers as for RT-PCR (Table 1), and fluorescence was quantified using an ABI PRISM 7900 apparatus (Applied BioSystems, Foster City, CA, U.S.A.). The expression of each gene was assayed in triplicate in a volume of 25  $\mu\text{l}$  containing 1 $\times$  SYBR green reagent and 1  $\mu\text{M}$  each gene-specific primer pair. After 10 min of denaturation at 95°C, 40 PCR cycles were performed as recommended by the manufacturer (95°C for 15 s, the appropriate primer annealing temperature for 30 s, and 72°C for 30 s). To calculate the number of transcripts present in original samples, plasmid DNA containing each amplicon was prepared using the Macherey-Nagel kit (Macherey-Nagel, Düren, Germany), quantified by UV absorbance spectroscopy, and linearized by restriction enzyme digestion (EC 3.1.21.5) (*EcoRI* for MtBC plasmids [Journet et al. 2002] and *NotI* for the two Topo plasmids [Promega Corp.]). Standard amplification curves were determined from duplicate samples of plasmid DNA at 10<sup>2</sup>, 10<sup>3</sup>, 10<sup>4</sup>, 10<sup>5</sup>, 10<sup>6</sup>, and 10<sup>7</sup> copies for each assay. To verify amplifi-

cation of each specific target cDNA, a melting-curve analysis was included at the end of each PCR run according to the thermal profile suggested by the manufacturer (95°C for 15 s, the appropriate primer annealing temperature for 15 s, and 95°C for 15 s). The generated data were analyzed by SDS 2.2 software (Applied Biosystems). For all amplification plots, the baseline data were set with the automatic cycle threshold ( $C_T$ ) function available with SDS 2.2, by calculating the optimal baseline range and threshold values by using the Auto $C_T$  algorithm (SDS 2.2 User Manual). Candidate-gene expression data from real-time RT-PCR were plotted as  $2^{(40-C_T)}/10$ , as described by Czechowski and associates (2004), and normalized against the constitutive reference gene determined in this study. According to the manufacturer's instructions, a  $C_T$  value of  $\geq 39$  corresponds to nonspecific amplification marking the limits of detection, which is equivalent to an expression threshold of 0.2. In order to compare effects of plant genotype on fungal gene expression, gene expression data from real-time RT-PCR obtained with the three mutated plants (*dmi1*, *dmi2/Mtsym2*, and *dmi3/Mtsym13*) were transformed in  $\log_2$  for positive values, then plotted against  $\log_2$  of fungal gene expression in wild-type *J5 M. truncatula*.

### Statistical analysis.

Data from real-time RT-PCR analyses of each fungal gene were compared in replicate RNA batches (three biologically independent experiments) from *G. intraradices* quiescent or germinating spores, or *G. intraradices*-inoculated roots of wild-type *J5 M. truncatula*, or of the three symbiosis-defective mutants and were statistically compared using one-way analysis of variance and Tukey's pairwise comparisons test with PAST (Hammer et al. 2001). Homogeneity of variance was tested with Levene's test.

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## AUTHOR-RECOMMENDED INTERNET RESOURCES

Institut National de la Recherche Agronomique (INRA) MYCOCALC program: [www.dijon.inra.fr/mychintec/Mycocalc-prg/download.html](http://www.dijon.inra.fr/mychintec/Mycocalc-prg/download.html)  
 Medicago EST navigation system (MENS) website: [medicago.toulouse.inra.fr/Mt/EST/](http://medicago.toulouse.inra.fr/Mt/EST/)