Membrane steroid-binding protein 1 induced by a diffusible fungal signal is critical for mycorrhization in *Medicago truncatula*

Hannah Kuhn[1], Helge Küster[2] and Natalia Requena[1]

[1]Botanical Institute, University of Karlsruhe and Karlsruhe Institute of Technology, Plant–Microbe Interactions Group, Hertzstrasse 16, D–76187, Karlsruhe, Germany; [2]Institute of Plant Genetics, Unit IV - Plant Genomics, Leibniz Universität Hannover, Herrenhäuser Str. 2, D-30419 Hannover, Germany

Author for correspondence:
Natalia Requena
Tel: 0721 608 4626
Email: natalia.requena@bio.uka.de

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Summary

- Arbuscular mycorrhiza (AM) is a mutualistic biotrophic association that requires a complex exchange of signals between plant and fungus to allow accommodation of the mycosymbiont in the root cortex. Signal exchange happens even before physical contact, activating the plant symbiotic program.
- We investigated very early transcriptional responses in *Medicago truncatula* to inoculation with *Glomus intraradices* and identified four genes induced by diffusible AM fungal signals before contact. Three of them were previously shown to be mycorrhiza induced at later stages of the symbiosis, while *MtMSBP1*, encoding a membrane-bound steroid-binding protein, is a novel mycorrhizal marker.
- Expression analyses in plants defective in the symbiotic receptor kinase DMI2 allowed discrimination of two different signaling cascades involved in the perception of the diffusible signals. Thus, while some of the genes are activated in a DMI2-dependent manner, the induction of one of them encoding a proteinase inhibitor is DMI2-independent.
- Downregulation of *MtMSBP1* by RNAi led to an aberrant mycorrhizal phenotype with thick and septated appressoria, decrease number of arbuscules and distorted arbuscule morphology. This provides genetic evidence that MtMSBP1 is critical for mycorrhiza development. We hypothesize that MtMSBP1 plays a role in sterol homeostasis in the root.

Introduction

Plants have evolved to interact with different microorganisms to establish different types of associations ranging from parasitic to mutualistic. The immune system of the plant has developed to recognize signal/pattern molecules to discriminate between deleterious and beneficial microorganisms (Zipfel & Felix, 2005). Arbuscular mycorrhiza (AM) fungi belong to the latter group and plants forming this association specifically recognize their symbiotic partners and consequently modify their genetic programs to accommodate them (Parniske, 2004). The molecular bases of this recognition process are starting to be understood. They point to common signaling pathways shared with other microbe–plant associations like the nodule symbiosis and to specific signaling pathways commonly elicited by all arbuscular mycorrhizal fungi (Catoira *et al.*, 2000; Stracke *et al.*, 2002; Liu *et al.*, 2003, 2007; Hohnjec *et al.*, 2005; Kistner *et al.*, 2005; Gutjahr *et al.*, 2008; Güther *et al.*, 2009). As only a few plant families are nonmycorrhizal (Smith & Read, 1997), it is clear that most plants have maintained the necessary machinery to associate with these obligate symbionts for at least 450 million yr (Redecker *et al.*, 2000). However, some plants such as the model *Arabidopsis thaliana* are missing some of the essential plant signaling elements required for mycorrhization (Lévy *et al.*, 2004; Mitra *et al.*, 2004; Kevei *et al.*, 2005). Future research will disclose how many other components are missing or altered in nonmycorrhizal plants.

Similar to the rhizobial symbiosis, where the plant is the first to 'talk' to its bacterial partner with a cocktail of flavonoids that induces expression of the *nod* genes, in the arbuscular mycorrhizal symbiosis the fungus reacts to the

production of strigolactones by the roots with hyphal branching (Akiyama *et al.*, 2005). This phenomenon is known to occur upon recognition of compatible host root exudates (Giovannetti *et al.*, 1993; Buee *et al.*, 2000), although it is not sufficient to induce appressoria formation. However, in contrast to the nodule symbiosis, where the identification of the lipochitin–oligosaccharide signals (Nod factors; NF) has led to considerable advances in the dissection of the signal cascade that initiates the symbiotic program in the plant, it is not known how AM fungi 'talk' to their host plants. From genetic experiments using legume mutants impaired in nodule symbiosis we know that receptors for NF perception (LYK3, NFP in *Medicago truncatula*, NFR1 and NFR5 in *Lotus japonicus*) are not involved in perception of mycorrhizal signals (Wegel *et al.*, 1998; Limpens & Bisseling, 2003; Madsen *et al.*, 2003; Radutoiu *et al.*, 2003). Therefore it is likely that AM fungal signals are of a different chemical nature. Nod factor perception culminates in the activation of at least four specific transcription factors that mediate all NF-induced transcriptional responses (Kaló *et al.*, 2005; Smit *et al.*, 2005; reviewed in Oldroyd & Downie, 2008). Although, these transcription factors are also dispensable for arbuscular mycorrhiza symbiosis (Catoira *et al.*, 2000; Oldroyd & Long, 2003; Hirsch *et al.*, 2009), at least seven of the intermediate signaling components are essential for establishment of the AM symbiosis (Catoira *et al.*, 2000; Kistner *et al.*, 2005). This common pathway has been referred as the SYM (symbiotic pathway) and contains a receptor like kinase, nuclear pore proteins, nuclear ion channels, a calcium/calmodulin-dependent protein kinase and an associated protein (Cyclops) of unknown function (for recent reviews see Parniske, 2008; Oldroyd *et al.*, 2009). In particular, three SYM proteins have been extensively studied and shown to be early transmitters in both symbioses. In *M. truncatula* they were named DMI proteins (does not make infections). Molecular and genetic analyses of these genes showed that *DMI1* (*POLLUX* in *L. japonicus*), encoding a cation-permeable channel, and *DMI2* (*SYMRK* in *L. japonicus*), encoding a receptor like kinase, are hierarchically situated above *DMI3*, the calcium and calmodulin-dependent protein kinase (Wais *et al.*, 2000). Both proteins are required to induce a calcium oscillation signal that is decoded by DMI3 (Kaló *et al.*, 2005; Smit *et al.*, 2005). While it was long known that NF induce calcium spiking as one of the earliest responses in root hairs, only recently it was shown that AM fungi elicit both, a transient cytosolic calcium elevation (Navazio *et al.*, 2007) and calcium oscillation (Kosuta *et al.*, 2008). In the first case, this happens in response to a fungal diffusible signal constitutively produced (Navazio *et al.*, 2007), while calcium oscillation appears to be dependent on the presence of branched strigolactone-induced hyphae (Kosuta *et al.*, 2008). Although the AM fungal diffusible constitutive signal was able to transcriptionally activate all DMI genes (Navazio *et al.*, 2007), it is not known whether the transmission of this signal travels through this cascade. By contrast, calcium oscillation in response to a diffusible signal released by branched AM hyphae was shown to be dependent on DMI2 and DMI1 (Kosuta *et al.*, 2008).

There are several examples of the divergence in the perception of NF and AM fungal signals, indicating the existence of more than one pathway for the establishment of root symbioses. These pathways are likely to share some components and to be triggered by more than one signal. Thus, while the symbiotic activation of the early nodulin *MtENOD11* by NF is DMI dependent, its activation in AM symbiosis is DMI-independent when triggered by a diffusible fungal signal (Kosuta *et al.*, 2003) or DMI-dependent when triggered by appressorium formation (Chabaud *et al.*, 2002). Similarly, the induction of lateral root formation (LRF) induced by both NF and diffusible AM fungal signals requires different elements of the DMI cascade (Oláh *et al.*, 2005). Hence, while NF-induced LRF requires all three components, DMI3 is dispensable for LRF triggered by a diffusible AM fungal signal. This indicates that if calcium oscillations are produced and needed for LRF during mycorrhiza symbiosis, they are not decoded by DMI3. Interestingly, induction of LRF in rice has been recently shown to be independent of the DMI1 and DMI3 rice orthologues (Gutjahr *et al.*, 2009). This could suggest that calcium oscillation might not be necessary for perception of the diffusible AM fungal signal that induces LRF.

In this work we describe a novel *M. truncatula* gene (*MtMSBP1*) that is early induced by a diffusible AM fungal signal produced by branched hyphae. The inactivation of this gene leads to changes in the development of the fungus inside the cortex with the appearance of abnormal appressoria and aberrant or collapsing arbuscules. The transcriptional analysis of *MtMSBP1* and of the other identified early marker genes clearly shows that at least two different signaling cascades exist for perception of an AM fungal diffusible signal. Even more, it may suggest the possibility that more than one fungal diffusible signal might exist. Thus, while one of these signals would travel independently of the receptor-like kinase DMI2, the perception of the second signal would be DMI2 dependent.

Materials and Methods

Biological material

Medicago truncatula seeds from the genotype Jemalong A17 (wild type) or dmi2 mutant TR25, containing *PMtENOD11*:GUS (Catoira *et al.*, 2000; Chabaud *et al.*, 2002), were germinated as described in Boisson-Dernier *et al.* (2001). *Glomus intraradices* DAOM 181602 (Schenck & Smith, 1982) was maintained in the *in vitro* system described by Bécard & Fortin (1988) with *Agrobacterium rhizogenes* transformed carrot rots in bicompartmental Petri plates as described in St-Arnaud *et al.* (1996).

Plasmid construction

The red fluorescent marker DsRED1 under the control of the constitutive *Arabidopsis* Ubiquitin10 promoter (P*Ubq10*) (Limpens *et al.*, 2005) was cloned (*Kpn*I) into the binary Gateway vectors pKGWFS7 and pK7FWG2,0 (both Karimi *et al.*, 2002) resulting in the promoter reporter vector pPGFPGUS-RedRoot and the plasmid pCGFP-RedRoot for *C*-terminal GFP fusion and expression under the 35S promoter of the cauliflower mosaic virus (CMV). Genomic DNA of *M. truncatula* was isolated with the DNeasy plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. A *MtMSBP1* promoter fragment of 1518 bp was PCR-amplified from genomic DNA using the primers: P*MtMSBP1*-forward/reverse. The coding region of *MtMSBP1* was amplified from cDNA using the primers *MtMSBP1*-forward/reverse. The promoter fragment was cloned into pPGFPGUS-RedRoot and the coding region of *MtMSBP1* was recombined into pCGFP-RedRoot by using the pENTR/DTOPO and the Gateway system (Invitrogen). The RNAi constructs were created using the binary Gateway vector pK7GWIWG2D(II) for hairpin RNA expression under the control of the CMV 35S promoter (Karimi *et al.*, 2002). *MtMSBP1* DNA region −18 bp to +186 bp (with respect to the ATG position, where A is position +1) was amplified from genomic DNA using the primers *MTMSBP1*-RNAi-forward/reverse and recombined into pK7GWIWG2D(II). Ten hairy root explant lines were obtained and from those five expressing GFP stably after several rounds of subcultivation were further used for real-time polymerase chain reaction (PCR) analyses. All primers used in this manuscript are listed in the Supporting Information, Table S1.

Generation of transgenic *M. truncatula* hairy root lines

Generation of hairy roots was conducted after the protocol of Boisson-Dernier *et al.* (2001) with *A. rhizogenes* ARqua1 (Quandt *et al.*, 1993) using either *M. truncatula* wild-type or *dmi2* mutant plants. Hairy roots emerging from the root section were explanted and propagated stably on M medium (Bécard & Fortin, 1988) supplemented with 25 mg l^{-1} kanamycin for selection and decreasing concentrations of 400, 200 and 0 mg l^{-1} augmentin (Amoclav; Hexal, Holzkirchen, Germany) to eliminate *A. rhizogenes*. For each transformation at least 50 plants were used and between 10 and 20 lines per construct were propagated and investigated for stability of the introduced T-DNA.

In vitro mycorrhizal colonization of *M. truncatula*

We developed an *in vitro* system for mycorrhization of *M. truncatula* roots. Hyphae growing in the distal part of the *D. carota–G. intraradices* system described by St-Arnaud *et al.* (1996) were used as a source of mycorrhizal inoculum. Whole *M. truncatula* plants or hairy root explants were mycorrhized in this system either in liquid or solid M medium, respectively. For whole plant inoculation extraradical mycelium was grown in liquid medium as described before (Helber & Requena, 2008). After 10 d, three to five seedlings were planted into openings on the plate lid close to the border of the bipartite plate. Plants were incubated at 25°C with a photoperiod of 16 h of light.

Hairy root explants were colonized on solid M medium. To achieve this, extraradical hyphae were allowed to grow on a cellophane membrane on top of the M medium. Hyphal passage to the distal compartment was facilitated by a swell (a ramp or step from the distal compartment to the border with the proximal compartment) and a thin layer of M medium poured on top of the cellophane membrane (Fig. 1e). To synchronize the colonization of hairy root explants, extraradical hyphae were grown *c.* 7 d until the fungal compartment was covered with hyphae. Hairy root explants were then added and covered with some water and a second cellophane membrane was set on top to maximize the contact area between fungus and root.

Appressoria enrichment, time course experiment and sandwich assay of mycorrhizal colonization

Appressoria-enriched root fragments from either whole *M. truncatula* plants or hairy root explants were isolated, cut and frozen in liquid nitrogen for subsequent RNA extraction and microarray analysis. Growth of extraradical hyphae towards the roots was monitored every day under the stereo microscope and root fragments (2–3 mm long) that came in contact with hyphae were excised and stored in RNAlater solution (Qiagen) at 4°C. Similar root fragments from uncolonized roots were collected as control. Ten different plates for each treatment were used. The time-course of mycorrhizal colonization was carried out in solid medium using *M. truncatula* hairy root explants. Mycorrhiza formation was monitored periodically by staining with black ink (Vierheilig *et al.*, 1998). Samples for RNA extraction were taken after 3, 6, 8, 13 and 17 d. Roots from at least three plates were pooled at each time-point, frozen in liquid nitrogen and stored at −80°C.

To test the influence of fungal diffusible signals on plant gene expression a sandwich system was employed where *M. truncatula* hairy root explants were separated from germinated spores (3 d of growth) or extraradical hyphae (7 d of growth) by a permeable cellophane membrane. For inoculation with germinated spores *G. intraradices* spores were isolated as described earlier (Helber & Requena, 2008). Spores of 40 plates were collected in 2 ml of water and 200 μl of spore suspension were applied directly onto the cellophane. When triggered with extraradical hyphae, mycelium was prepared as described earlier. Plates were incubated for up

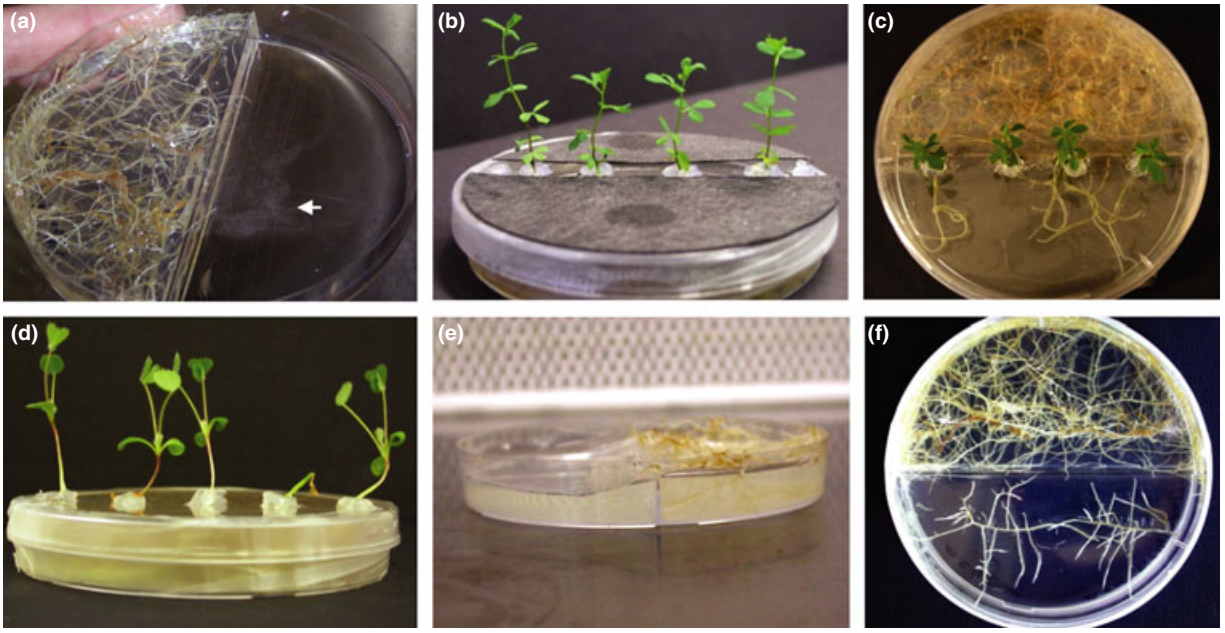


Fig. 1 *In vitro* system for mycorrhization of seedlings and hairy root explants. (a) Hyphae of *Glomus intraradices* (arrow) growing in liquid M medium, originating from *Daucus carota* mycorrhizal root cultures located in the distal compartment. (b) Side view of *Lotus japonicus* seedlings, planted into liquid medium for inoculation with *G. intraradices* hyphae. The openings in the lid of the Petri dish were sealed with sterile silicon grease. To protect the roots from light the lid of the Petri dish was covered with black paper. (c) Top view of same experimental setup as in (b) but without black paper. (d) The same experimental setup as in (b) and (c) but with *Medicago truncatula* plantlets. (e) Bi-partite Petri dish prepared for inoculation of hairy root explants. The medium swell (a ramp or step from the distal compartment to the border with the proximal compartment) next to the compartment border facilitates colonization of the empty compartment by *G. intraradices hyphae*. (f) *Medicago truncatula* hairy root explants are colonized by *G. intraradices* hyphae originating from *D. carota roots* as described in (e).

to 5 d and roots of at least three plates were sampled after 1 d and 5 d. The roots of each time-point were pooled, frozen in liquid nitrogen and stored at −80°C. In all experiments control mock-inoculated roots were also isolated.

Real-time reverse-transcription polymerase chain reaction (RT-PCR) and RT-PCR analyses

Total RNA was isolated with RNeasy plant mini kit (Qiagen) according to the manufacturer's protocol. The integrity of RNA was visualized on denaturing agarose gels and quantification was done spectrophotometrically. A 1-μg sample of total RNA was digested with DNAse I (Amplification grade; Invitrogen) and reverse transcription was performed in a 20-μl reaction using the Superscript II reverse transcriptase and OligodT Primer (Invitrogen).

Transcript levels of genes were determined on a Bio-Rad iCycler MyIQ using MESA Green qPCR MasterMix Plus (Eurogentec, Köln, Germany) and a primer concentration of 0.2 pmol μl^{-1} in 25-μl reactions. A 1-μl sample of a 1 : 10 dilution of single-strand cDNA was used as a template. For each primer set gradient PCR was performed to determine the optimal annealing temperature. Final PCR conditions were selected as follows: 3 min at 95°C (no cycling); 30 s at 95°C, 30 s at 56°C and 30 s at 72°C (40 cycles). Every real-time PCR run was done at least twice in three biological replicates with three technical replicates per reaction. Melt curve data was collected after each run. Ten-fold dilution standard curves were taken for each primer set to determine the efficiency of amplification. Threshold cycle location as well as further data analysis was performed with the IQ5 optical system software version 2.0 using the ΔΔCt method based on Vandesompele *et al.* (2002) and taking the primer efficiencies into consideration (Bio-Rad). Transcript levels were normalized to the constitutively expressed translation elongation factor 1-alpha *MtTEF1α* (TC106470, DFCI *M. truncatula* gene index) (Hohnjec *et al.*, 2003; Wulf *et al.*, 2003; Nyamsuren *et al.*, 2007).

For RT-PCR, RNA was isolated from *M. truncatula* roots, shoot, leaves, flowers and pods and cDNA was synthesized as described earlier. The RT-PCR conditions were the same as described for real-time RT-PCR with the exception that only 30 cycles were programmed.

Gene-specific primers used were as follows: MtMSBP1 forward/reverse; *MtTEF1α*-forward/reverse; *MtEnod11*-forward/reverse. Primers for TC106351, TC107197 and TC112474 were as published by Liu *et al.* (2007).

Histochemical analysis of tissue

Staining for glucuronidase (GUS) activity was performed according to Hohnjec *et al.* (2003) based on the protocol of

Jefferson *et al.* (1987). Fungal structures were visualized by ink staining (Vierheilig *et al.*, 1998).

Quantification of mycorrhizal colonization

Quantification of mycorrhizal colonization was done as described by Trouvelot *et al.* (1986) using the program MYCOCALC (http://www2.dijon.inra.fr/mychintec/Mycocalc-prg/download.html). In addition to the values described by Trouvelot *et al.* (1986) abundance of intraradical hyphae in the mycorrhizal part of the root system (*i*%) was determined as described for arbuscule abundance and calculated equally.

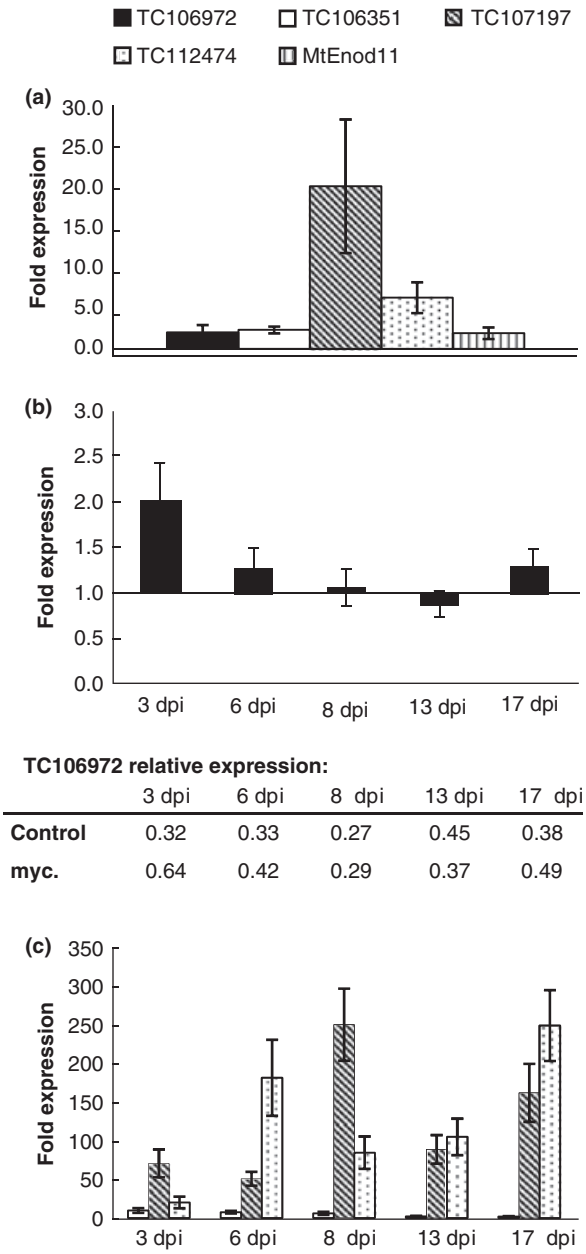
Microscopy

Roots for long observations were prepared for microscopy as described by (Genre *et al.*, 2005). Microscopy was done either with a conventional epifluorescence microscope Zeiss AxioImager Z1 or the Laser Scanning microscopes Zeiss LSM 510 meta and Leica TCS SP5 with water immersion objectives ×60 or ×63.

Results

Identification of *M. truncatula* early-induced genes during arbuscular mycorrhizal symbiosis

Plants can perceive AM fungi at very early stages of the symbiosis even before physical contact (Kosuta *et al.*, 2003; Weidmann *et al.*, 2004). This perception is manifested in changes in gene expression that presumably prepare the plant for the accommodation of the fungal partner. We attempted here to identify new marker genes of early mycorrhization in *M. truncatula.* Using a novel experimental setup under total root sterility seedlings were mycorrhized *in vitro* in a bicompartmental plate system shown in Fig. 1a. Here AM hyphae are grown in liquid M medium and seedlings are 'planted' on the plate. We succeeded with this system to colonize several plants, such as *M. truncatula*, *L. japonicus* (Fig. 1b–d) or *Solanum lycopersicum*. Appressoria formation was monitored under the stereomicroscope after removing the plants from the system and root segments (2–3 mm) harboring contact points or appressoria were harvested and RNA extracted. Transcriptional analysis carried out with the Mt16kOLI1 microarrays (Hohnjec *et al.*, 2005) yielded *c.* 500 genes regulated vs noncolonized root segments. From those, 237 were upregulated (data not shown), 90 of them at least 2.4 fold (log2-ratio $M \geq 1.25$), using a *P*-value threshold of 0.05 (Table S2). Based on the original data set, several candidate genes induced in appressorium-enriched samples with induction levels between 0.5 and 5 were validated by real-time PCR. From those, four genes were further verified in different biological replicates



TC106972 relative expression:

	3 dpi	6 dpi	8 dpi	13 dpi	17 dpi
Control	0.32	0.33	0.27	0.45	0.38
myc.	0.64	0.42	0.29	0.37	0.49

Fig. 2 Quantitative real time reverse-transcription polymerase chain reaction (RT-PCR) gene expression analysis of selected genes. (a) Expression of selected early induced genes in appressoria-enriched root fragments. Time-course expression analysis of TC106972 (b), and TC106351, TC107197 and TC112474 (c) during hairy root mycorrhization with *Glomus intraradices.* Roots were sampled after 3, 6, 8, 13 and 17 d post infection (dpi). In both experiments the expression is compared with equally treated non-colonized roots and expressed as a fold-number of induction or expression relative to *MtTEF1a*. The experiments were done with three biological replicates. The PCR reactions were repeated twice with three technical replicates. Standard deviation of the average is given.

(Fig. 2a). Three of these genes were previously identified as mycorrhiza specific but their induction was only studied at later stages (TC106351, TC112474, TC107197; Liu *et al.*,

2007; Wulf *et al.*, 2003). The first two encode proteinase inhibitors while the third is a protein of unknown function designated specific tissue protein 2. The fourth gene identified was not previously reported as mycorrhiza induced (TC106972) and showed similarity to membrane steroid-binding proteins. The expression of the early marker gene, *MtENOD11* was also included in the analysis as reference (Chabaud *et al.*, 2002) and showed a similar induction to TC106972 in appressoria-enriched samples.

To assess whether the induction of the four genes studied was maintained at later stages of the symbiosis, we carried out a time-course analysis of expression. Mycorrhized *M. truncatula* hairy root explants were monitored periodically (Fig. 1e,f) and their expression analysed at different time-points. In this system, mycorrhization is very efficient and many appressoria were observed at 3 d post infection (dpi). At 6 dpi intraradical mycelium and some arbuscules are observed, while 8–13 d correspond to high abundance of arbuscules. At 17 dpi vesicles can be observed. The time-course analysis of TC106972 expression showed that the gene was induced at 3 d post inoculation, an appressoria-rich stage, confirming the results shown above (Fig. 2b). Induction was of a similar magnitude albeit a bit lower, indicating a dilution effect owing to the use of whole roots instead of appressoria-enriched samples. Interestingly, the expression of TC106972 returned to basal levels after 6 d and remained low for the rest of the time course. This was in contrast to the other three genes analysed, which remained induced at later stages. However, whereas TC107197 and TC112474 expression was further increased at later stages of mycorrhization, expression of TC106351 was highest at earlier time-points of the symbiosis (Fig. 2c)

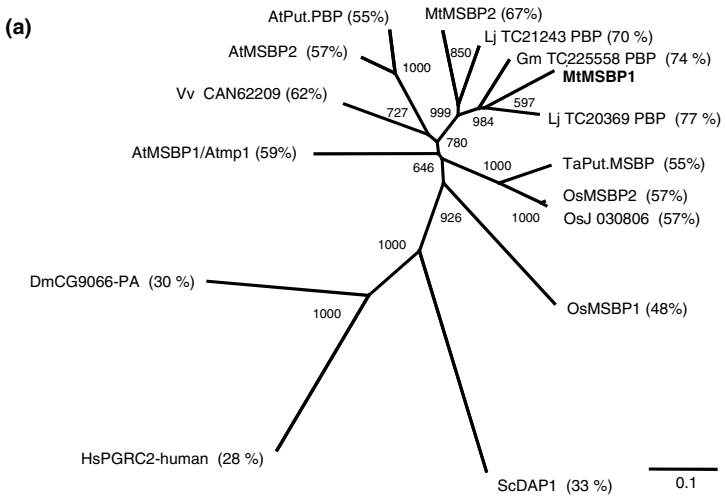
MtMSBP1 encodes a membrane steroid-binding protein

BLASTX analyses showed that TC106972 has homology to a family of proteins called membrane steroid-binding proteins widespread in the plant kingdom but also conserved in animals, insects and fungi. A member of this family in *A. thaliana* was recently identified as MSBP1, therefore we designated the TC106972 as *MtMSBP1* (Yang *et al.*, 2005). Phylogenetic analysis of several eukaryotic members of this protein family showed that MtMSBP1 clusters together with the plant proteins. The closest homology (77% identity) was found to the *L. japonicus* progesterone-binding protein (PBP) TC20369 followed by a homologue from *Glycine max* (74% identity) (Fig. 3a). A second *M. truncatula* membrane steroid-binding protein was found in the TIGR database (corresponding to TC95008 and TC95009), which shares 67% identity with MtMSBP1. We designated it as MtMSBP2. Interestingly, a membrane steroid-binding protein from *L. japonicus* (Lj TC21243) has been recently found to be induced at early stages of mycorrhiza formation with *Gigaspora margarita* and repressed in arbuscule-containing cells (M. Güther and P. Bonfante, pers. comm.). Lj TC21243 showed higher similarity to MtMSBP2 than to MtMSBP1. Therefore, we tested whether *MtMSBP2* followed a similar trend of early induction. However, MtMSBP2 was not found to be mycorrhiza-induced at any stage of the symbiosis (data not shown). This could indicate that perhaps the *L. japonicus* protein encoded by Lj TC21243 is the functional orthologue of MtMSBP1.

MtMSBP1 is located in chromosome 6 and it is part of the artificial chromosome AC157779. The corresponding gene consists of two exons (a 410 bp exon containing the start codon, and 660 bp exon containing the stop codon) separated by an intron of 4132 bp. The intron position is conserved in *A. thaliana*, between the amino acids Ser104 and Arg105. *MtMSBP1* cDNA was deduced to encode a polypeptide of 235 amino acids with a calculated molecular mass of 25.8 kDa and a theoretical isoelectric point of 4.55. MtMSBP1 contains a noncleavable signal peptide (amino acids 1–40), a single *N*-terminal transmembrane region (amino acids 15–37) and a conserved cytochrome b5-like heme/steroid binding domain (72–169 amino acids) Pfam motif PF00173 (Fig. 3b). A conserved tryptophan residue (Trp155), a putative binding site of progesterone, is also present in MtMSBP1. The protein structure indicates an *N*-terminal anchoring to a membrane and a catalytic domain for steroid binding, typical of membrane-associated steroid receptors (Falkenstein *et al.*, 1996; Meyer *et al.*, 1996; Cenedella *et al.*, 1999).

MtMSBP1 localizes to the ER

The presence of a noncleavable signal peptide and a *N*-terminal transmembrane region suggests the localization of the protein at a membrane. The WOLF PSORT program (http://wolfpsort.org, Horton *et al.*, 2006) predicts in addition a *C*-terminal endoplasmic reticulum (ER) membrane retention signal, ADKE, indicating that this protein could be localized to the ER. While animal and fungal homologues of MSBP have been found to localize to the ER, including perinuclear localization (Min *et al.*, 2005), the *A. thaliana* MSBP1, surprisingly, was found to localize at the plasma membrane (Yang *et al.*, 2005). In order to determine precisely the subcellular localization of MtMSBP1 in *M. truncatula* we cloned the coding region of *MtMSBP1* into the binary vector pCGFP-RR resulting in a *C*-terminally GFP-tagged version of the protein under the control of the CMV 35S promoter. The *M. truncatula* hairy roots were generated and the localization of the protein was analysed by epi-fluorescence and confocal microscopy. The GFP signal in young root hairs was found at a perinuclear localization as well as in membranous structures projecting from the nucleus towards the root hair tip. These structures most



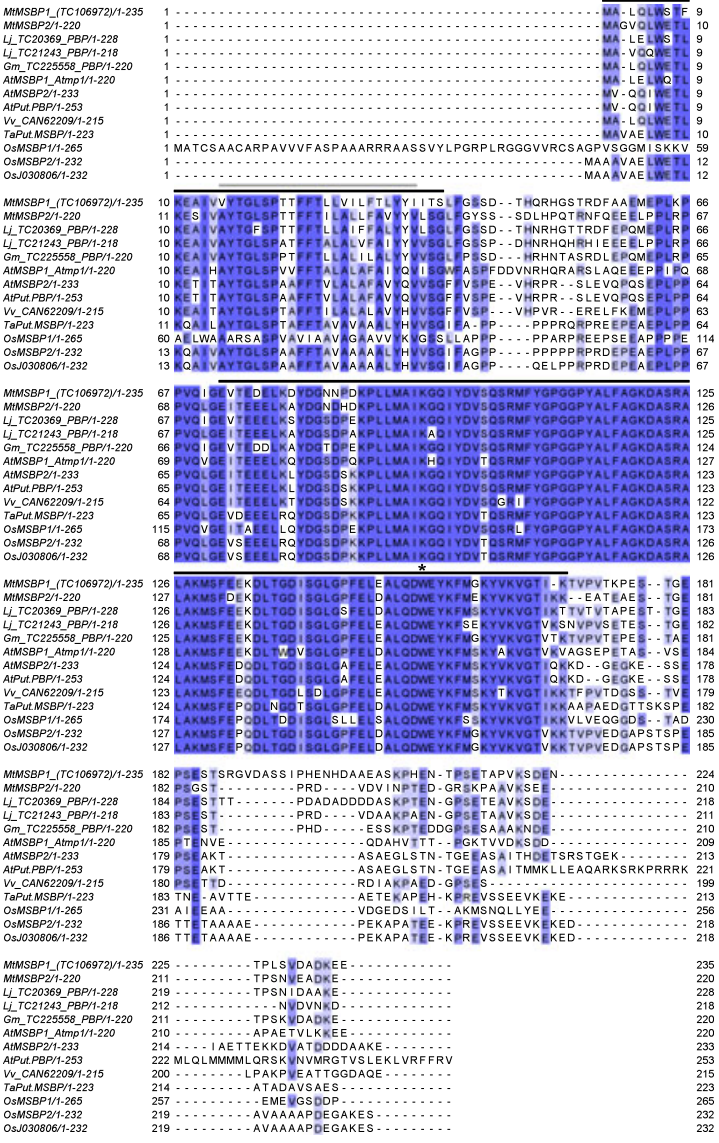


Fig. 3 MtMSBP1 belongs to a widespread protein family present in all eukaryotes. (a) Phylogenetic relationship among MtMSBP1 and other membrane steroid-binding proteins. Bootstrap neighbor-joining tree of MtMSBP1 and related proteins. The phylogenetic tree was calculated with CLUSTAL X 1.81 (Thompson *et al*., 1997) using the neighbor-joining method and bootstrapping. The following full-length (derived) amino acid sequences were used: *Medicago truncatula* (MtMSBP1: TC106972; MtMSBP2: TC95008), *Lotus japonicus* (TC20369; TC21243), *Glycine max* (TC225558), *Arabidopsis thaliana* (AtMSBP1: NP_200037; AtMSBP2: NP_190458), *Vitis vinifera* (CAN62209), *Triticum aestivum* (ABB90550), *Oryza sativa* (OsMSBP1: NP_001064992; OsMSBP2: NP_001064993), *Homo sapiens* (PGC2-human (Membrane-associated progesterone receptor component 2), O15173), *Drosophila melanogaster* (CG9066-PA (NP_573087)) and *Saccharomyces cerevisiae* (DAP1 (CAA97876)). Number in brackets represent percentage identity to MtMSBP1. (b) Multiple amino acid sequence alignment of MtMSBP1 with related proteins. The alignment was calculated with CLUSTAL X 1.81 and colored in JALVIEW (Waterhouse *et al*., 2009) according to the proteins percentage identity. First black line, predicted signal anchor; gray line, predicted transmembrane helix; second black line, conserved cytochrome b5-like heme/steroid-binding domain. Asterisk indicates conserved tryptophan residue necessary for heme binding.

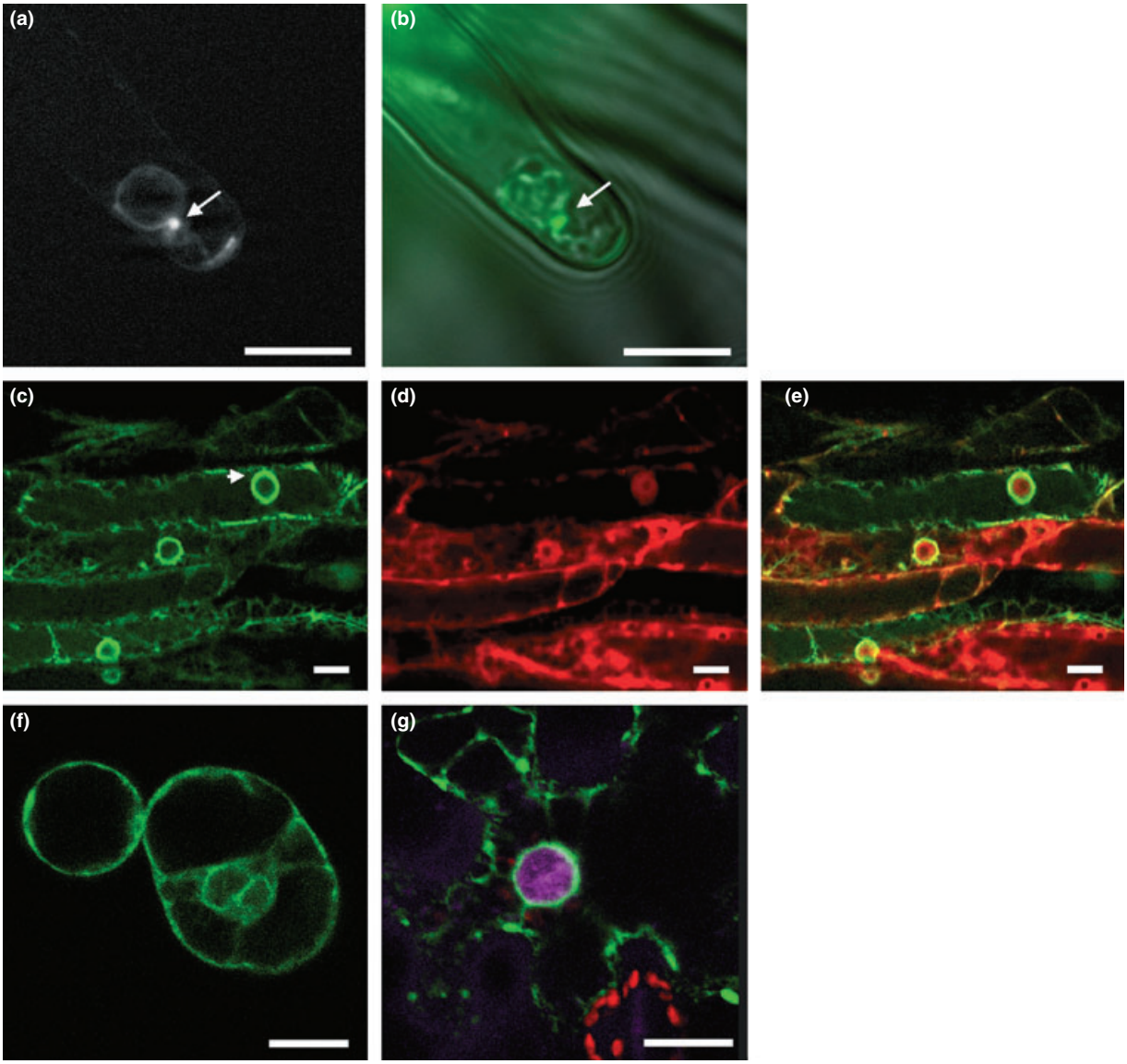


Fig. 4 Subcellular localization of MtMSBP1-GFP. (a–g) Expression of *MtMSBP1-GFP* under control of the CMV 35S promoter in *Medicago truncatula* hairy root explants. (a,b) Localization of MtMSBP1-GFP in root hairs. The GFP fluorescence can be observed in the endoplasmic reticulum (ER) around the nucleus (arrow) and in the cytoplasmic ER projections connecting to cortical ER of the root hair tip. (c–e) Localization of MtMSBP1-GFP in cortical root cells observed under the confocal microscope in a single z-layer. Green fluorescence (c) corresponds to MtMSBP1-GFP, while red fluorescence (d) shows the reporter DsRED under the control of the ubiquitin10 promoter as a control. (e) The overlay of the two fluorescence channels. MtMSBP1-GFP can be observed at the ER network as well as in the perinuclear ER (arrowhead) but never inside the nucleus. By contrast, DsRED localizes in the cytoplasm and inside the nucleus while absent in the nucleolus. (f,g) Expression of the fusion protein MtMSBP1-GFP in heterologous systems. (f) In *Arabidopsis thaliana* protoplasts and (g) in *Nicotiana benthamiana* epidermal cells. The GFP fluorescence corresponding to MtMSBP1 localizes in both cases to the ER. In epidermal cells of *N. benthamiana* red fluorescence from chloroplast of the stomata guard cells and the cell nucleus stained with 4,6-diamidino-2-phenylindole (DAPI) in blue can also be observed. Bars, 10 μm.

likely correspond to the different ER populations: nuclear, cortical and cytoplasmic connections to cortical ER (Fig. 4a,b). Very often MtMSBP1 accumulated as a bright spot around the nucleus (Fig. 4a). Confocal microscopy in cortical cells clearly showed that the GFP signal was localized in the ER network, including the nuclear envelope, but not inside of the nucleus. By contrast, DsRED signal, constitutively expressed, could be observed in the cytoplasm and within the nucleus (Fig. 4c,d,e). The localization of MtMSBP1-GFP at the ER mirrors the localization of the construct *P35S*:GFP-HDEL containing the HDEL ER retention signal shown recently by Genre *et al.* (2005). To test whether the ER localization pattern of MtMSBP1was *M. truncatula* specific, protoplasts of *A. thaliana* and epider-

mal cells of *N. benthamiana* were transformed with the same construct. In both cases MtMSBP1-GFP showed ER localization, including perinuclear localization (Fig. 4f,g).

MtMSBP1 expression analyses

We analysed the expression of *MtMSBP1* in different plant tissues by RT-PCR (Fig. 5a) and *in silico* using the *M. truncatula* gene expression atlas (http://bioinfo.noble.org/gene-atlas/). The gene was expressed in all tissues, root, shoot, leaves, flowers and pods. In contrast to the homologue in *A. thaliana*, which is very low expressed in roots compared with other tissues (Yang *et al.*, 2005), *MtMSBP1* is similarly highly expressed in all tissues analysed. According to the *in silico* data, expression is about twofold higher in roots than in other tissues (Benedito

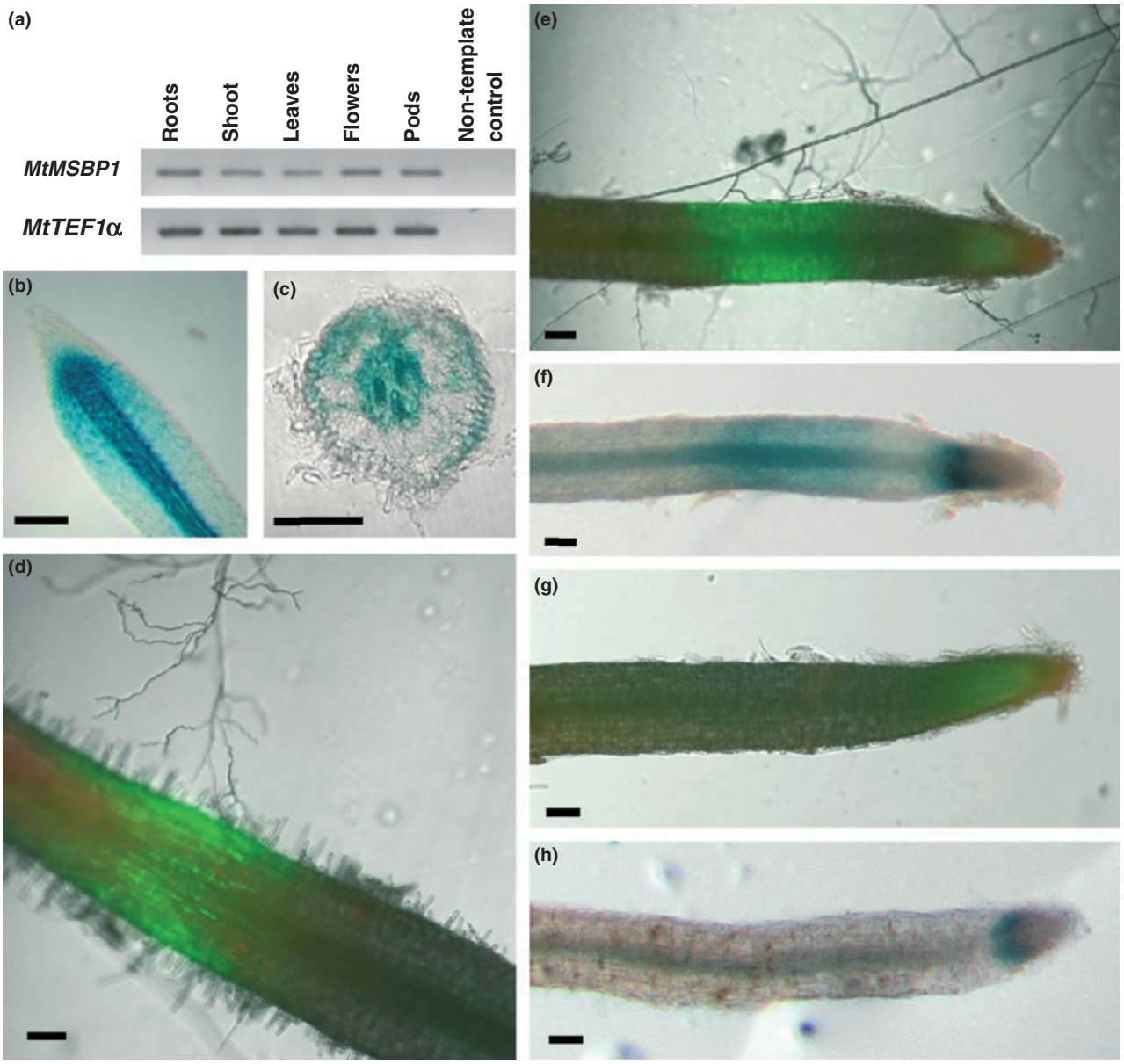


Fig. 5 Expression analysis of *MtMSBP1*. (a) RT-PCR analysis of *MtMSBP1* in different *Medicago truncatula* tissues. DsRED1 is expressed under the control of the constitutively active *Arabidopsis thaliana* Ubiquitin 10 promoter and was used as a selection marker. (b–g) Expression of the P*MtMSBP1:GFP–GUS* fusion in hairy root explants visualized by GFP-fluorescence (d,e,g) or histochemical staining for GUS-activity. (b,c,g,h) Non-mycorrhizal hairy root explants. (b) Root tip cleared with KOH. (c) Cross-section of non-mycorrhizal root. (d,e,f) Expression of the P*MtMSBP1:GFP-GUS* construct in hairy roots mycorrhized *in vitro*. (d,e) Promoter activity indicated by GFP fluorescence in roots in contact with highly branched hyphae. (e) The same root as in (e) was stained for β-glucuronidase activity. (f) GFP-fluorescence and (g) GUS staining of a corresponding nonmycorrhizal control hairy root. Bar, 100 μm.

et al., 2008). To analyse *MtMSBP1* expression in roots in more detail, a 1518 bp promoter region upstream of the ATG was isolated from genomic DNA and fused to the reporter genes *GFP-GUS*. Transgenic stable hairy root explants were produced and both *in vivo* fluorescence as well as histochemical GUS staining were carried out. The β-glucuronidase activity showed that *MtMSBP1* was constitutively expressed in the central cylinder as well as in the apical meristem (Fig. 5b,c,h). In some cases expression could be seen in epidermal and cortical cells in regions very close to the apical meristem (Fig. 5c).

The temporal and spatial induction of *MtMSBP1 in vivo* during mycorrhization was studied in stably transformed hairy root explants expressing the *GFP-GUS* reporter genes by periodical monitoring using fluorescence microscopy. It could be observed that *MtMSBP1* was often induced in root areas in contact with branched hyphae originating from runner hyphae running parallel to the root (Fig. 5d,e), in contrast to control roots (Fig. 5g). The GUS staining confirmed this result and showed that in addition to the *MtMSBP1* constitutive expression in the vascular cylinder and in the apical meristem observed in control roots, a mycorrhiza-specific GUS activity was induced in root areas in contact with branched hyphae (Fig. 5f). To determine more precisely in which root cells *MtMSBP1* was induced upon hyphal contact, we used Z-stack confocal microscopy. The GFP fluorescence could be seen in epidermal and subepidermal cells in the surrounding area of *G. intraradices* branched hyphae (Fig. 6a–f). However, we cannot exclude induction in other root areas as the fluorescence in deeper tissue layers is difficult to detect.

A secreted AM fungal signal triggers *MtMSBP1* expression before hyphal contact

To analyse the onset of mycorrhiza-induced expression, time-lapse video microscopy was carried out. Transgenic *M. truncatula* hairy root explants expressing the *GFP-GUS*

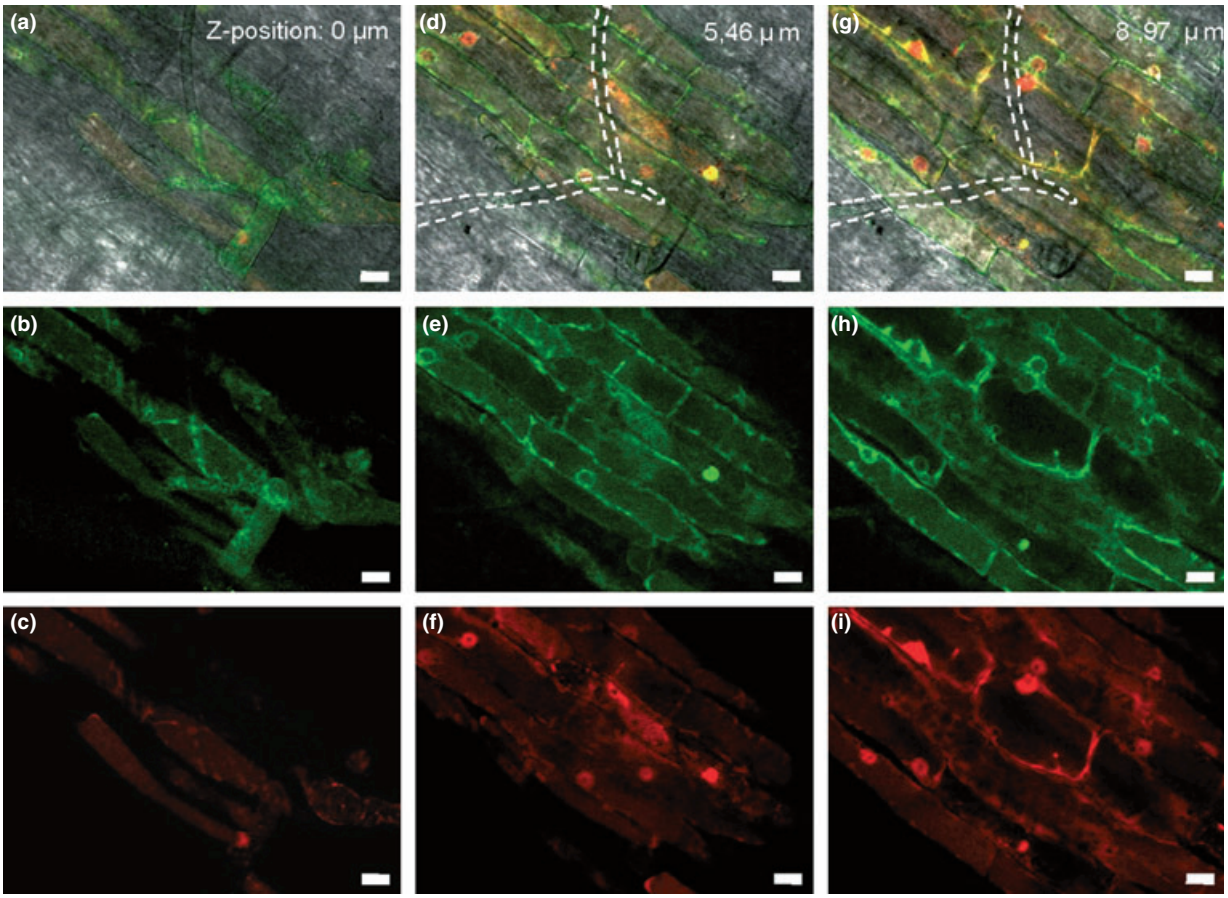


Fig. 6 Cellular localization of *MtMSBP1* expression in mycorrhizal tissue. Expression of the P*MtMSBP1:GFP–GUS* promoter construct in hairy root explants inoculated with *Glomus intraradices*. The GFP fluorescence indicates *MtMSBP1* promoter activity while DsRED1 is expressed under control of the constitutively active *Arabidopsis thaliana* Ubiquitin 10 promoter. (a–i) The z-stack of a confocal micrograph at the site of hyphal branching (arrow). (a–c) Top view of the root epidermal cells. (d–f) and (g–i) single z-layers 5.46 µm and 8.97 µm deeper in the root tissue showing intense GFP fluorescence in epidermal and subepidermal cells. The branching hypha is indicated by a dashed line. Bar, 10 µm.

reporter cassette under the control of the *MtMSBP1* promoter were mycorrhized. Root colonization was monitored with fluorescence microscopy. During a period of *c.* 3.5 h, it could be observed that a short hypha emerged from a runner hypha extending parallel to the root. This short hypha continued growing and branching towards the rhizodermis to form an appressorium. In that time, the promoter of *MtMSBP1* was activated in epidermal/subepidermal cells in the vicinity of the branching hypha. Surprisingly, expression was induced before hyphal contact to the root, pointing to a secreted diffusible signal. GFP could be after 2 h and 10 min (Fig. 7 and Video S1) indicating quick diffusion, perception and transduction of the fungal signal. This fact prompted us to investigate whether the expression of *MtMSBP1* could also be induced if there was no direct contact between fungus and plant, and signals could pass through a diffusible membrane. A 'sandwich system' was used placing a cellophane membrane between fungus and plant. Transgenic hairy root explants were triggered either with germinating spores or with extraradical hyphae, and

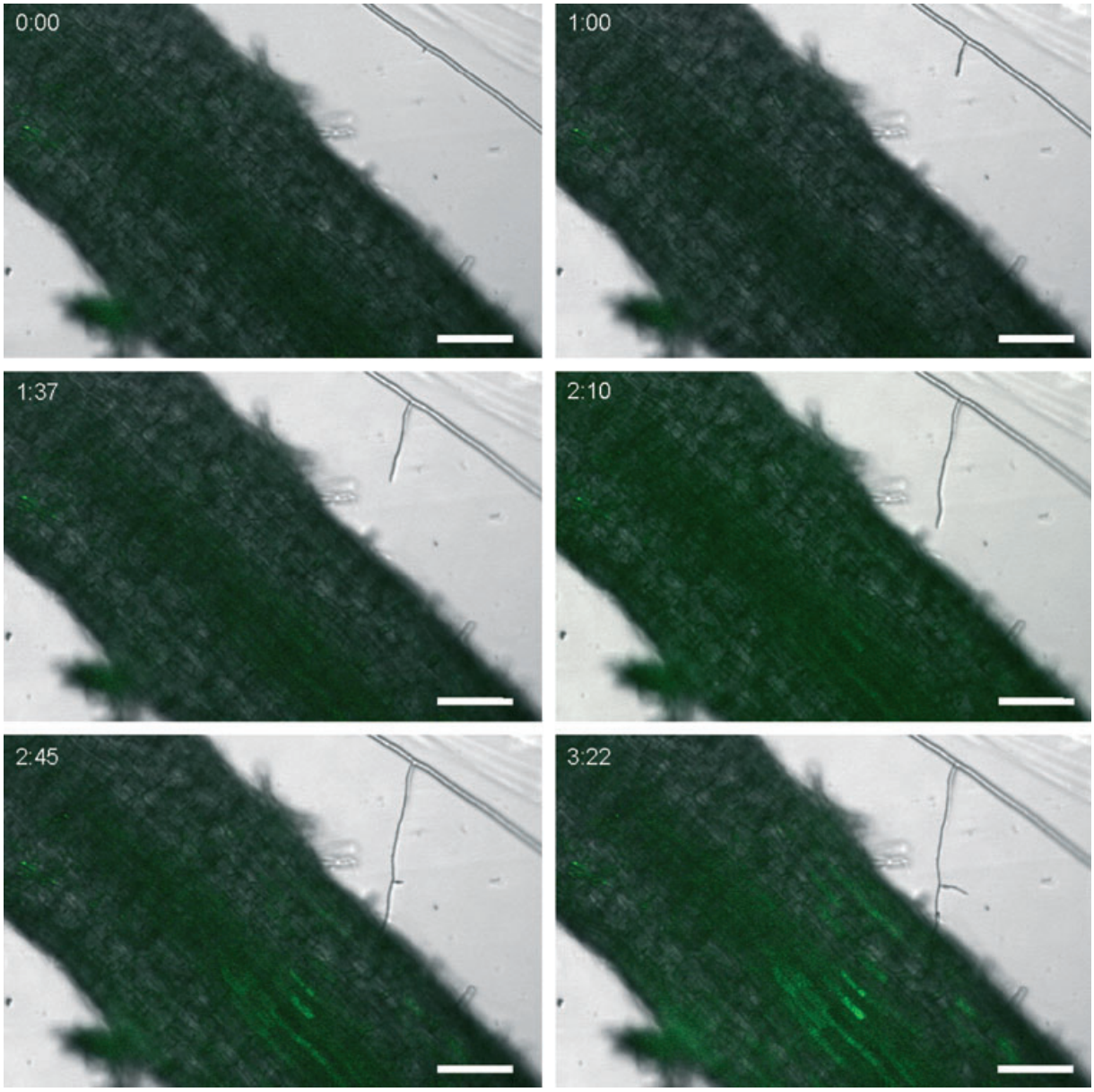


Fig. 7 Dynamic of mycorrhiza-induced expression of *MtMSBP1*. Time-lapse GFP expression study of *MtMSBP1* expression during early stages of mycorrhiza formation. Expression of the P*MtMSBP1:GFP–GUS* construct in hairy root explants inoculated with *Glomus intraradices*. *MtMSBP1* promoter activity, indicated by green GFP fluorescence, rises as branched hyphae, originating from a runner hypha, approach the root surface. Relative time is given in hours and minutes. Bar, 100 μm.

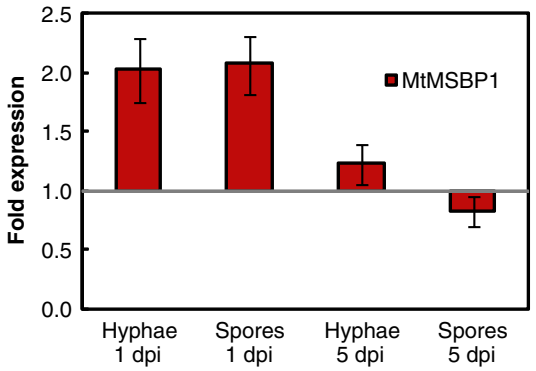


Fig. 8 Induction of *MtMSBP1* expression by diffusible molecules from *Glomus intraradices*. Quantitative real time polymerase chain reaction (PCR) showing expression of *MtMSBP1* in hairy root explants of *Medicago truncatula* triggered either by germinated spores or hyphae of *G. intraradices* placed behind a cellophane membrane after 1 d of 5 d of co-incubation. Expression values are compared with those of equally treated noninduced control hairy root explants at same time-points and normalized to *MtTEF1α*. The experiment was done with three biological replicates. The PCR reactions were repeated twice with three technical replicates. Standard deviation of the average fold-expression is given.

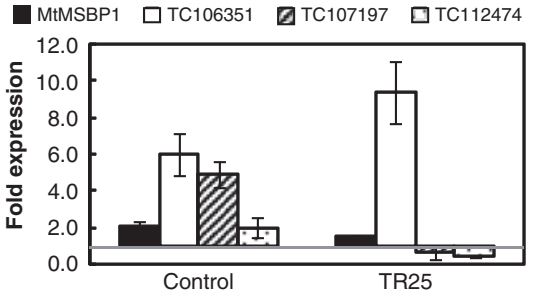


Fig. 9 Fold-expression of marker genes after diffusible signal induction in wild type and *dmi2-1* (TR25) hairy roots. Fold expression of *MtMSBP1*, TC106351, TC107197 and TC112474 was analysed by quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) in wild-type background control hairy root explants and *dmi2-1* (TR25) hairy root explants after 1 d of co-culture with extraradical hyphae separated by a cellophane membrane. The expression of samples induced by *Glomus intraradices* is compared with fungal free samples which were treated equally. Expression values were normalized to *MtTEF1α*. The experiment was done with three biological replicates. The PCR reactions were repeated twice with three technical replicates. Standard deviation of the average fold-expression is given.

expression was analysed by real-time PCR after 1 d or 5 d of contact. Indeed, results showed that both fungal propagules produce a secreted diffusible signal able to cross through the cellophane membrane and to induce the expression of *MtMSBP1* (Fig. 8). Interestingly, this induction was, in both cases, transient, coinciding with results from the time-course analysis (Fig. 2b).

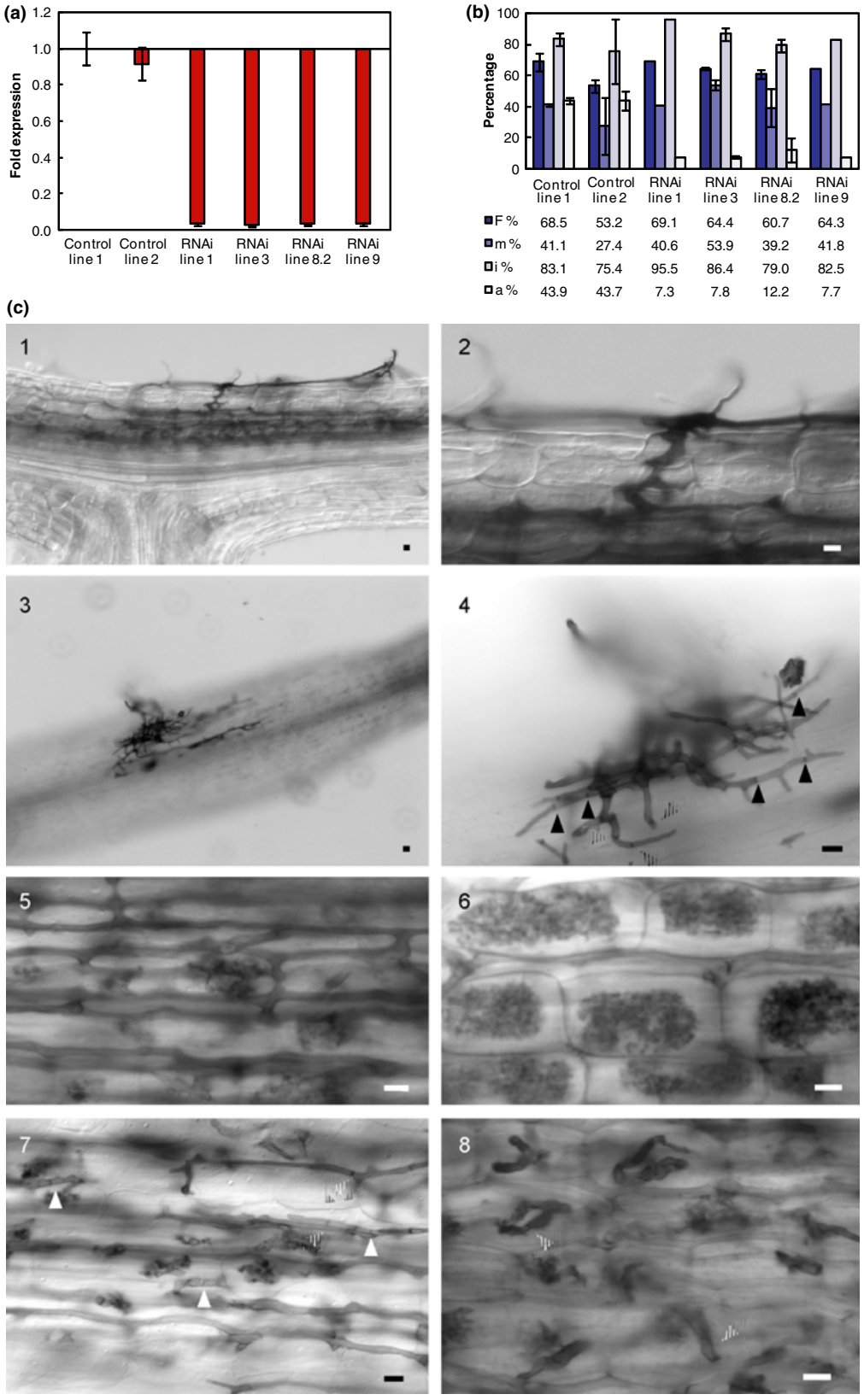
Diffusible AM fungal signal/s travel through two different signaling pathways

It is known that mycorrhiza formation requires an intact symbiotic pathway involving the receptor-like kinase DMI2 and the ion channel protein DMI1 to induce a calcium oscillation signal. Recently, it has been shown that a diffusible signal produced by branched hyphae of AM fungi is able to induce this calcium oscillation and that DMI2 and DMI1 are required for the perception of this signal (Kosuta *et al.*, 2008). Therefore, we addressed the question whether the induction of *MtMSBP1* and the other mycorrhiza early-induced genes was caused by a diffusible signal and whether this signal was travelling through the SYM pathway. As shown in Fig. 9, all genes analysed were induced in *M. truncatula* hairy roots explants with wild-type background when triggered by extraradical hyphae separated by a cellophane membrane. However, with the exception of TC106351, this induction was abolished (TC107197 and TC112474) or reduced (*MtMSBP1*) when *dmi2-1* mutant TR25 hairy roots explants were used. This result shows unambiguously that two signaling cascades are functioning to perceive AM fungal signals.

Inactivation of *MtMSBP1* results in a mycorrhiza-defective phenotype

Our work indicated that *MtMSBP1* was transcriptionally induced by a diffusible AM fungal signal. This result indicates that its induction might be significant for the development of the symbiosis. To genetically test this hypothesis we used RNA interference and analysed the phenotype of hairy root explants for mycorrhiza formation. We obtained

Fig. 10 Reduced *MtMSBP1* expression results in an aberrant mycorrhizal phenotype. Deficiency of MtMSBP1 in stable transgenic hairy root explants harboring P*35S*-MtMSBP1-RNAi constructs. (a) Fold-expression of *MtMSBP1* was analysed by quantitative real time polymerase chain reaction (PCR) in control hairy root lines 1 (transformed merely with *Agrobacterium rhizogenes*) and 2 (transformed with control GFP construct) and four independent transgenic lines indicating that *MtMSBP1* RNA interference was successful. (b) Colonization level was analysed according to Trouvelot *et al.* (1986) after ink and vinegar staining of roots 12 d post infection (dpi) with *Glomus intraradices*. In addition to the parameters *F*% (frequency of mycorrhiza in the root system), *m*% (intensity of the mycorrhizal colonization in mycorrhizal parts of the root system), *a*% (arbuscule abundance in the mycorrhizal parts of the root system) the value for *i*% (intraradical hyphae abundance in mycorrhizal parts of the root system) was determined using MYCOCALC software (MYCOCALC, see Trouvelot *et al.*, 1986). Note that *a*% is reduced in RNAi lines compared with control lines. (c) Ink stain of mycorrhizal roots often revealed an abnormal appressoria and arbuscule morphology in *MtMSBP1* RNAi lines (3, 4, 7 and 8) compared with control lines (1, 2, 5 and 6) hairy roots. Increased septation was often observed in hyphae developing in RNAi transgenic lines (arrowheads). Bar, 10 μm).



several stable transgenic hairy root explant lines with different levels of gene downregulation. All lines grew similar to the control lines and showed no developmental defects under non-symbiotic conditions (data not shown). Five hairy root lines were analysed by PCR, and four were selected for mycorrhizal analyses based on the highest degree of RNA inactivation. The expression levels of *MtMSBP1* in the four RNAi hairy root lines analysed were strongly reduced with respect to several control hairy root lines (transformed only with *A. rhizogenes* or with other control plasmids) (Fig. 10a). The expression of *MtMSBP2* coding for the second isozyme was checked in all lines and it was not significantly affected (data not shown). Transgenic RNAi hairy root lines were mycorrhized *in vitro* and analysed at 12 dpi by ink staining. Results showed that the frequency of mycorrhizal colonization in RNAi lines was similar to that from control lines (Fig. 10b). However, several important phenotypic differences were observed. Appressoria from RNAi lines often developed clumps of highly septated hyphae that ended in aborted infections (Fig. 10c). Nevertheless, infection succeeded in other areas of RNAi lines and then the fungus developed intracellular hyphae, arbuscules and vesicles. However, the number of arbuscules was strongly reduced in RNAi lines (80% reduction, Fig. 10b). Even more significant was that the abundance of collapsing arbuscules and septated hyphae was strongly increased with respect to the control lines (Fig. 10c).

Discussion

The establishment and maintenance of a mutualistic symbiosis between plants and microbes is a complex process that requires constant signal exchange between both partners to avoid defense reactions that would jeopardize the association. In the arbuscular mycorrhizal symbiosis this signal exchange occurs before physical contact in both directions. Strigolactones released from host roots have been shown to induce hyphal branching in AM fungi (Akiyama *et al.*, 2005) and one or more diffusible signal molecules from AM fungi elicit transient cytosolic calcium elevation in host plant cells, induction of symbiosis related genes and calcium oscillation (Kosuta *et al.*, 2003, 2008; Weidmann *et al.*, 2004; Navazio *et al.*, 2007). However, the nature of the fungal signal(s) is still unknown. The perception of AM fungal signals travels at least partly through the SYM pathway, but evidence of a second signal cascade has been recently shown (reviewed in Oldroyd *et al.*, 2009). Here we present further evidence of the existence of at least two different signal cascades in *M. truncatula* for perception of AM fungal diffusible signals.

In this work we identified *MtMSBP1* in a screening for early mycorrhiza induced genes together with other early markers (TC107197, TC106351 and TC112474). *MtMSBP1* encodes a protein of the recently identified membrane-bound steroid-binding protein family that in plants is associated with the regulation of cell elongation (Yang *et al.*, 2005). Although this gene is highly expressed in roots during asymbiosis in the central cylinder and in the meristem of the root apex, its expression is transiently induced two- to three-fold at very early stages of the AM symbiosis by a diffusible signal. The induced expression was observed in epidermal and subepidermal cells in the vicinity of approaching hyphae and in appressorium-enriched root areas. The induction in appressorium-enriched samples coincides with an induction of the early marker gene *MtENOD11* (Journet *et al.*, 2001). *MtENOD11*, a repetitive hydroxyproline-rich protein, has been widely used as space and time marker of mycorrhization (Chabaud *et al.*, 2002; Kosuta *et al.*, 2003). Similarly to the mycorrhizal-induced expression of *MtMSBP1* described in this work, *MtENOD11* is induced in root areas underneath branched hyphae (Kosuta *et al.*, 2003). This might indicate that not all hyphae from AM fungi are able to produce (enough) signal(s) inducing expression of these two genes. Therefore it is likely, that the prestimulation of branching by plant strigolactones not only facilitates docking of the fungus to the root but also makes AM fungal hyphae competent for symbiosis.

In our work here, time-lapse video microscopy and cellophane experiments clearly showed that *MtMSBP1* expression was induced before fungal contact. This shows that the branching hypha produces a secreted fungal compound, able to pass through a cellophane membrane, that triggers expression within 3 h. This rapid transcriptional induction suggests that the signal perception is transmitted through a pre-established signal cascade, perhaps involving phosphorylation. To date, only the SYM signal cascade mentioned above has been genetically defined for the perception of AM fungal signals. However, evidence that a second cascade might exist is accumulating. Thus, while *MtENOD11* is activated upon perception of a diffusible signal in mutant plants defective in three genes of the SYM pathway (DMI2, DMI1 and DMI3) (Kosuta *et al.*, 2003), calcium oscillation induced by a diffusible signal from AM fungi requires DMI2 and DMI1 (Kosuta *et al.*, 2008). Additional indication of an alternative signaling cascade to the SYM route has been recently observed in rice (Gutjahr *et al.*, 2009). The work presented here provides further indication of two different signal cascades operating in this symbiosis. The evidence is based on the differential activation of these target genes among wild-type and mutant plants defective in the symbiotic receptor kinase DMI2. In contrast to wild-type plants, that are able to perceive the AM-diffusible fungal signal/s and activate the four marker genes studied here, in DMI2 defective plants only one of the marker genes (TC106351) is clearly activated, indicating the existence of a DMI2-independent

pathway. In addition to two signaling cascades, it is possible that more than one fungal signal might exist. Thus, one of these signals would travel through the SYM pathway and the second through a yet unidentified route. Biochemical analyses will help to discern about the existence of more than one AM fungal signal.

In order to ascertain the role that MtMSBP1 plays in the mycorrhiza symbiosis we localized the protein subcellularly and inactivated it using RNA interference. Surprisingly, although the *A. thaliana* protein was described to locate to the plasma membrane, we clearly showed here that the *M. truncatula* protein localizes to the ER as it has been shown for yeast and animal homologues (Rohe *et al.*, 2009). Furthermore, heterologous localization of MtMSBP1 in *A. thaliana* protoplasts and in *N. benthamiana* leaves showed ER localization. This is consistent with steroids being molecules able to travel through biological membranes and to reach receptors within the cell lumen. Inactivation of MtMSBP1 by RNA interference showed a surprising but interesting phenotype. Thus, while we did not observe any morphological alterations in root development in the absence of mycorrhiza, the phenotype of the symbiotic structures within the root was affected. Hyphal penetration from appressoria was often aborted by septation of penetrating hyphae. Septation also occurred in intercellular hyphae but, most significantly, the morphology of arbuscules was distorted. Arbuscules developed much less frequently and with a crippled morphology in the RNAi hairy root lines, resembling the phenotype observed in the pea late mutant (RisNod24) or in the *M. truncatula* phosphate transporter mutant, *mtpt4-1*, when mycorrhized (Gianinazzi-Pearson, 1996; Lapopin *et al.*, 1999; Javot *et al.*, 2007). These results provide genetic evidence of the relevance of MtMSBP1 for the mycorrhiza symbiosis.

Membrane-bound steroid-binding proteins are a family of proteins first described in animals and later found in plants and fungi (Falkenstein *et al.*, 1996; Hand *et al.*, 2003; Iino *et al.*, 2007). In contrast to plasma steroid-binding proteins that locate to the cytoplasm and shuttle to the nucleus after steroid binding where they act as transcription factors, membrane-bound steroid-binding proteins contain a single transmembrane domain and are presumed to act through second messengers (Lösel *et al.*, 2003). In plants their role has been linked to the control of cell expansion/elongation as it was observed that hypocotyl length changes correlated to induction or repression of *AtMSBP1* (Yang *et al.*, 2005). However, recent work with the mammalian and yeast/fission yeast homologues have shown that this family of proteins might have a conserved role in the control of the sterol biosynthesis by binding and regulating ER-located cytochrome P450 enzymes (Mallory *et al.*, 2005; Hughes *et al.*, 2007). In this respect, Yang *et al.* (2005) also noted that in *A. thaliana*, from the 115 induced genes in transgenic lines over-expressing *AtMSBP1*, 13% were related to the steroid/sterol metabolism and signaling. The homeostasis of sterols appears to be of major importance for the establishment of root symbioses. Thus, experiments using sterol biosynthesis inhibitor fungicides have shown that the pattern of root sterols changes dramatically with application of fenpropimorph and this, in turn, impedes arbuscular mycorrhizal symbiosis (Campagnac *et al.*, 2008). Fenhexamide, another sterol biosynthesis inhibitor fungicide does not induce major changes in the sterol profile of the root, or modify the total colonization by *G. intraradices*, but arbuscule frequency is significantly reduced, phenocopying the effect of inactivation of *MtMSBP1*. During symbiosis with rhizobia, the DMI2 receptor kinase has been shown to interact with 3-hydroxy-3-methylglutaryl CoA reductase (MtHGMR1), an enzyme of the mevalonate biosynthetic pathway required for the synthesis of isoprenoids (Kevei *et al.*, 2007). Accordingly, application of lovastatin,which inhibits HGMR activity, or inactivation of HGMR by RNAi leads to a decrease in nodulation. We have evidence that DMI2 also interacts with HGMR during mycorrhiza formation (N. Rieger & N. Requena, unpublished results). *MtHMGR1* has also been described to be induced at early stages of mycorrhiza symbiosis (Liu *et al.*, 2003). Therefore, it appears that the control of sterol homeostasis is essential to accommodate mutualistic symbionts in the root. We propose that MtMSBP1 could be involved in this regulation by interaction with P450 ER enzymes during mycorrhiza formation.

In summary, we show here that AM fungi are able to produce diffusible signal(s) that are transmitted through two signaling pathways to induce an array of downstream effectors. We propose that one of these signals travels through the well-established SYM pathway or at least enters through this pathway requiring a functional DMI2. This diffusible signal activates TC107197, TC112474 and possibly *MtMSBP1*, novel markers for early mycorrhization. The second signal travels through a yet unknown signaling pathway to activate the specific mycorrhiza marker TC106351. At present, we cannot rule out whether these are all constitutive or induced signals. However, induction of *MtMSBP1* correlated with the presence of branched hyphae. Our results for the inactivation of MtMSBP1 show that AM fungal induction of *MtMSBP1* might be related to the need of altering the sterol metabolism to allow plasma membrane invagination and intracellular accommodation of the symbiont in the cortex.

Acknowledgements

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Supporting Information

Additional supporting information may be found in the online version of this article.

Methods S1 Microarray analysis.

Table S1 List of primers used in this work

Table S2 Upregulated genes from the microarray analysis showing at least 2.4-fold induction for a *P*-value ≤0.05, based on two biological replicates

Video S1 Dynamic of mycorrhiza-induced expression of *MtMSBP1*.

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