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An AM-induced, *MYB*-family gene of *Lotus japonicus* (*LjMAMI*) affects root growth in an AM-independent manner

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SUMMARY

The interaction between legumes and arbuscular mycorrhizal (AM) fungi is vital to the development of sustainable plant production systems. Here, we focus on a putative MYB-like (*LjMAMI*) transcription factor (TF) previously reported to be highly upregulated in *Lotus japonicus* mycorrhizal roots. Phylogenetic analyses revealed that the protein is related to a group of TFs involved in phosphate (Pi) starvation responses, the expression of which is independent of the Pi level, such as PHR1. GUS transformed plants and quantitative reverse transcription PCR revealed strong gene induction in arbusculated cells, as well as the presence of *LjMAMI* transcripts in lateral root primordia and root meristems, even in the absence of the fungus, and independently of Pi concentration. In agreement with its putative identification as a TF, an eGFP-LjMAMI chimera was localized to the nuclei of plant protoplasts, whereas in transgenic *Lotus* roots expressing the eGFP-LjMAMI fusion protein under the control of the native promoter, the protein was located in the nuclei of the arbusculated cells. Further expression analyses revealed a correlation between *LjMAMI* and *LjPT4*, a marker gene for mycorrhizal function. To elucidate the role of the *LjMAMI* gene in the mycorrhizal process, RNAi and overexpressing root lines were generated. All the lines retained their symbiotic capacity; however, RNAi root lines and composite plants showed an important reduction in root elongation and branching in the absence of the symbiont. The results support the involvement of the AM-responsive *LjMAMI* in non-symbiotic functions: i.e. root growth.

Keywords: arbusculated cells, legumes, putative-transcription factor, phosphate transporter, root architecture, symbiosis, *Lotus japonicus*.

INTRODUCTION

Arbuscular mycorrhizal (AM) symbiosis involves most land plants and a number of soil-born fungi, belonging to the ancient phylum Glomeromycota (Wang and Qiu, 2006). In this mutualistic association, the AM fungus improves the mineral nutrition of the plant with the uptake of several nutrients from the soil (e.g. phosphate and nitrogen), whereas the plant supplies its heterotrophic partner with sugars. This symbiosis is the result of a complex exchange of molecular information that starts in the rhizosphere before the partners come into physical contact (Bonfante and Requena, 2011; Kloppholz *et al.*, 2011; Maillet *et al.*, 2011), and continues during all steps of the colonization process (Parniske, 2008; Bonfante and Genre, 2010). Mycorrhizal functionality requires the presence of arbuscules, which are considered to be the site of the nutrient exchanges (Harrison, 2005). Arbuscules (a Latin term for 'little trees') are formed in the inner root cortex by repeated branching of an intracellular hypha. The mechanisms controlling their development are still unknown, although some of the genes involved have been recently identified in *Medicago truncatula* by reverse genetics. RNAi lines of the mycorrhizal-specific phosphate transporter MtPT4 were found to display arbuscular morphogenetical defects, as arbuscules degenerated prematurely (Javot *et al.*, 2007). Similarly, the expression of another *M. truncatula* gene, named *Vapyrin*, and of its homolog in *Petunia*, is essential

for arbuscule formation, as *Vapyrin* RNAi induced a marked decrease in fungal epidermal penetration, and blocked the process of cortical cell colonization (Feddermann *et al.*, 2010; Pumplin *et al.*, 2010).

The significant cell reorganization during root colonization is associated with important changes in the transcriptomic profile of AM roots. The pattern of gene expression of different root cell types during the colonization has been investigated by genome-wide transcriptome profiling, combined with quantitative real-time reverse transcription-PCR (qRT-PCR) on several model plants, including legumes (e.g. Hohnjec *et al.*, 2005; Liu *et al.*, 2007; Guether *et al.*, 2009; Hogekamp *et al.*, 2011; Gaude *et al.*, 2012) as well as non-legumes, like *Oryza sativa* (rice; Güimil *et al.*, 2005) and *Solanum lycopersicum* (tomato; Fiorilli *et al.*, 2009; Garrido *et al.*, 2010). In *Lotus japonicus*, more than 500 protein-coding genes were found to be differentially regulated during the arbuscular phase (Guether *et al.*, 2009). Interestingly, a common element of the transcriptomic analyses on *Medicago* or *Lotus* mentioned above (Liu *et al.*, 2003; Guether *et al.*, 2009; Hogekamp *et al.*, 2011; Gaude *et al.*, 2012) is the presence of a putative transcription factor, an MYB-like protein, among the most upregulated genes in arbusculated cells.

Transcription factors (TFs) represent 5% of the genome in Arabidopsis (Riechmann, 2000) and 5.9% in *Medicago* (Young *et al.*, 2011). However, Udvardi *et al.* (2007) reported that less than 1% of TF genes in the model legumes *Lotus* and *Medicago* have been genetically characterized. One of the few areas of legume biology where the role of TFs has been firmly established is the nitrogen-fixing symbiosis involving legumes and some soil bacteria, called rhizobia (Udvardi *et al.*, 2007), as has been confirmed by the sequencing of the entire *Medicago* genome. Here, nodule-enhanced expression was markedly higher among TFs (92 out of 1513) than among all genes, confirming the crucial role of TFs in nodule development (Young *et al.*, 2011). Starting with *Nin*, for 'nodule inception' (Schauser *et al.*, 1999), which was the first TF gene to be identified as crucial for nodule formation, many other TFs, such as the NSP proteins, have been identified in both *Lotus* (Nishimura *et al.*, 2002) and *Medicago* (Kaló *et al.*, 2005; Smit *et al.*, 2005), as essential determinants for nitrogen-fixing symbiosis (Udvardi *et al.*, 2007; Young *et al.*, 2011). On the contrary, information on TFs involved in AM symbiosis is limited to the detection of putative TFs in transcriptomic profiles of *Medicago* mycorrhizal roots (Liu *et al.*, 2003; Gaude *et al.*, 2012), with the exception of Hogekamp *et al.* (2011), who described the expression profile of two CAAT-box TFs during the colonization process, starting from the early contact phases.

With the final aim of understanding the regulatory mechanisms that govern plant–fungal interactions during AM symbiosis, we focused our research on the putative *Lotus* MYB-TF sequence found to be the second highest upregulated gene in the mycorrhizal roots of *L. japonicus*, the transcripts of which were localized to arbusculated cortical cells using laser microdissection technology (Guether *et al.*, 2009). We demonstrated that this gene belongs to the class of MYB-like TFs, and that the protein product is indeed located in the nucleus of active arbuscule-containing cells. The gene is related to the PHR (phosphate starvation response) and PSR (phosphorous starvation response) proteins involved in phosphate (Pi) starvation, and its expression is independent of Pi level, and partially correlates with that of *LjPT4*, the reference marker for mycorrhizal functionality. However, in addition to the expected location in arbusculated cortical cells, *GUS*-promoter constructs revealed a constitutive presence of the protein in the meristems of non-mycorrhizal roots and lateral root primordia. Because of its induction in root meristems and arbusculated cells, we called this gene *LjMAMI* for meristem and arbuscular mycorrhiza induced. As RNAi lines from both hairy roots and composite plants maintain their mycorrhizal capacities, but have a strong phenotype, characterized by decreased branching, differing from both the control and the overexpressing lines, we concluded that *LjMAMI* may have a dual role: on one hand it could act as a regulator of some mycorrhizal-responsive genes in arbusculated cells, and on the other it could be a player in the mechanisms that regulate root growth.

RESULTS

Gene isolation and phylogenetic analysis of *LjMAMI*

The 941–bp cDNA sequence of the MYB-like gene previously found to be strongly upregulated in arbusculated cells during AM symbiosis (Guether *et al.*, 2009) was obtained by 5′ and 3′ rapid amplification of cDNA ends (RACE; accession number HE801636). The corresponding genomic sequence spanned around 3700 bp on chromosome 1, and comprised five introns (Figure 1a). The predicted protein sequence was 233 amino acids long, with a molecular mass of 26.2 kDa and a predicted pI of 9.58. At the N terminus of the protein the Prosite database predicted a one-repeat MYB domain, which consists of three conserved helices (Figure 1b). The third helix of the one-repeat MYB domain is generally involved in DNA binding. Indeed, the conserved putative DNA binding sequence SHAQK(F/Y) (Lu *et al.*, 2002) was found in this region, with only one conservative mutation (A→L). PAIRCOIL predicted a coiled-coil motif in the center of the sequence, partially overlapping with the nuclear localization signal predicted by NETNES.

A comparison between LjMAMI and the currently available sequences of *L. japonicus* and *M. truncatula* MYB TFs led us to exclude the presence of closely related homologous genes, which could have arisen from duplication events.

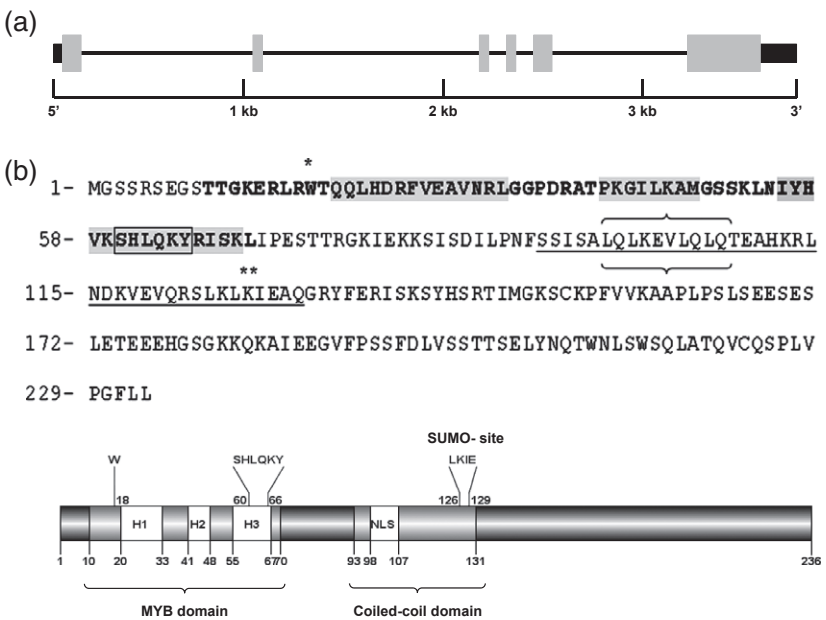

Figure 1. Features of *LjMAMI* and protein sequences.
(a) Schematic representation of the *LjMAMI* genomic sequence. Untranslated regions and exons are represented with black and gray bars, respectively.
(b) LjMAMI protein sequence, showing the putative coiled-coil region (underlined), the predicted nuclear localization sequence (brace brackets) and the MYB one-repeat domain (bold), with the three alpha helices in gray and the putative conserved DNA recognition motif boxed. Only one tryptophan out of the three generally conserved in one-repeat MYB genes is present in the sequence, as indicated by the asterisk. The predicted sumoylation lysine residue is indicated by two asterisks. A schematic representation of the protein is proposed in the lower part of the figure. H1, H2 and H3, MYB domain helices; NLS, nuclear localization signal; W, tryptophan conserved residue; SUMO-site, predicted sumoylated lysine.

Phylogenetic analysis (Figure 2) showed similarity between LjMAMI, and an *M. truncatula* MYB gene, previously shown to be upregulated during the first stages of AM symbiosis with several *Glomus* species (Liu *et al.*, 2003; Hogekamp *et al.*, 2011). Interestingly, among the currently available sequences, the most similar belonged to the GARP subgroup of MYB TFs (Feller *et al.*, 2011), and were related to Pi-starvation responses. The best-characterized gene of this group is *Arabidopsis thaliana PHR1* (*AtPHR1*), the DNA binding site of which has been identified as the imperfect palindromic motif GNATATNC (Rubio *et al.*, 2001), named P1BS. AtPHR1 has been shown to modulate the transcription of several genes according to Pi availability, whereas its own expression is independent of Pi levels (Rubio *et al.*, 2001; Nilsson *et al.*, 2007). AtPHR1 homologs have been identified in organisms ranging from algae (CrPSR1, Wykoff *et al.*, 1999) to monocots (OsPHR1-2, Zhou *et al.*, 2008) and dicots (PvPHR1, Valdés-López *et al.*, 2008).

Therefore, sequence analysis and phylogenetic comparisons suggest that LjMAMI might have a mycorrhizal-dependent role as a transcriptional regulator of Pi assimilation.

LjMAMI expression is independent of Pi level, but is correlated with AM colonization

On the basis of the phylogenetic analysis, LjMAMI was found to be related to a group of Pi-starvation proteins. To define whether or not its expression was dependent on Pi

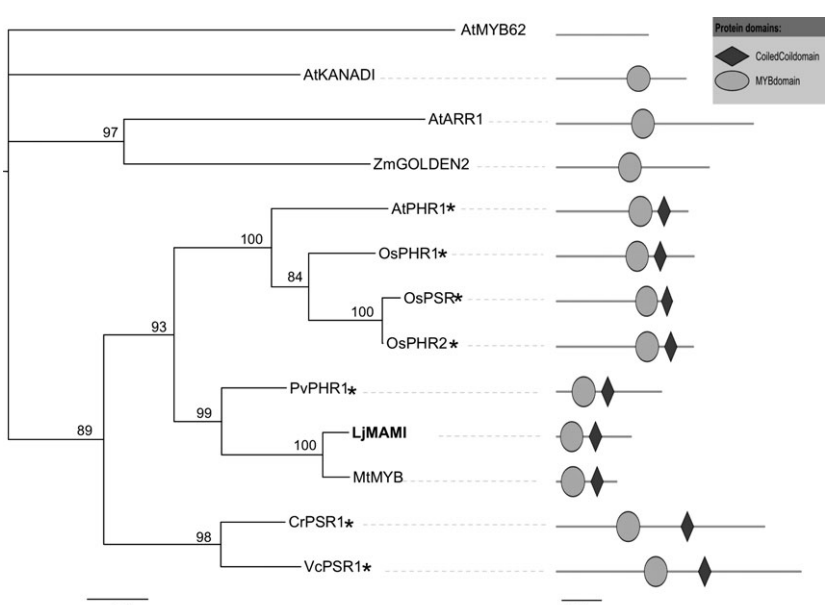

Figure 2. Phylogenetic tree of LjMAMI and related protein sequences. The most similar sequence to LjMAMI, MtMYB (AW585594.1), is still uncharacterized, whereas OsPSR (AAO72597), OsPHR1 (AK063486.1), OsPHR2 (AK100065.1), AtPHR1 (NP_194590.2), PvPHR1 (ACD13206.1), CrPSR1 (XP_001700553.1), VcPSR1 (XP_002951273.1), AtKANADI (NP_568334.1), ZmGOLDEN2 (NP_001105018.1) and AtARR1 (NP_566561.2) all belong to the GARP family of MYB TFs, and some of them (*) are related to Pi starvation responses. AtMYB62 (NP_176999) is an out-group. Numbers above branches represent Bayesian posterior probability (BPP) values.

concentration, we analyzed the *LjMAMI* expression levels on mycorrhizal roots growing on 2 μM, 20 μM or 2 mM Pi. In Guether *et al.* (2009) it was already demonstrated that *LjMAMI* is highly expressed at the concentration of 20 μM Pi, a condition that allows for the development of excellent symbiosis. However, it is known that 2 mM Pi is a high but non-toxic level that causes a reduction, but not the total suppression, of fungal colonization (Branscheid *et al.*, 2010; Breuillin *et al.*, 2010), whereas a lower concentration of Pi, such as 2 μM, is not expected to have an impact on the establishment of symbiosis.

The roots were sampled after 28 days, their colonization percentages were quantified and *LjMAMI* expression was evaluated by qRT-PCR. The transcript profiles closely followed the colonization percentages (Figure 3a and b).

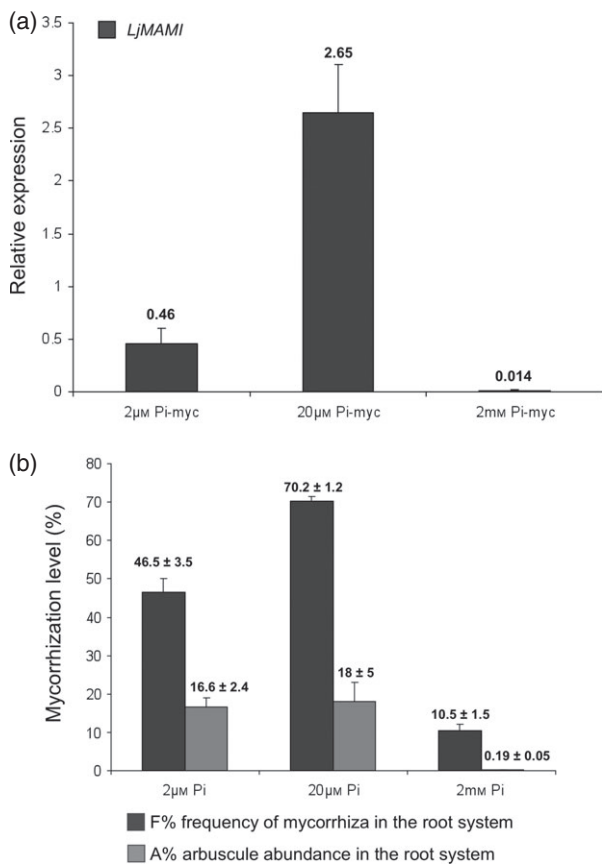

Figure 3. *LjMAMI* expression is independent of phosphate (Pi) concentration, but depends on arbuscular mycorrhizal (AM) colonization.
(a) qRT-PCR on mycorrhizal roots treated at three Pi levels after 28 days post-inoculation (dpi). *LjMAMI* expression was very low at 2 mM Pi, where the AM colonization was limited to some patches; the expression values were higher at 2 μM and peaked at 20 μM Pi. Values are the means of three replicates with SEs.
(b) Frequency of mycorrhizal hyphae and arbuscule abundance of the same samples, after trypan blue staining. Under these conditions, the levels of mycorrhization were favoured at 20 μM Pi with respect to 2 μM Pi. One hundred root fragments of 1–cm lengths were analyzed for each sample. The mean values and SEs of two biological replicates of each treatment are shown.

LjMAMI expression was very low at 2 mM, where the mycorrhization was limited to some patches, higher at 2 μM, where the fungus colonized a more important part of the root systems, but peaked at 20 μM Pi. Here, the frequency of mycorrhization and the arbuscule abundance were favored with respect to treatment with 2 μM Pi.

In conclusion, changes in the expression of *LjMAMI* observed in the mycorrhizal roots at different Pi concentrations mirror the differences in the colonization values. The gene expression does not linearly depend on Pi concentration, with transcript values being much more important at 20 μM Pi than at 2 μM or 2 mM Pi. The results provide further support to the strong AM dependency of the gene.

Histochemical GUS staining reveals mycorrhizal- and Pi-independent *LjMAMI* expression in specific root tissues

To get a general view of the *LjMAMI* expression pattern in the whole root system, the *LjMAMI* promoter was fused to the reporter gene β–glucuronidase (*GUS*). This construct was introduced into *Lotus* roots by *Agrobacterium rhizogenes*-mediated transformation (Stougaard *et al.*, 1987). Composite plants were grown with or without *Gigaspora margarita* inoculation and fertilized with either 20 μM or 2 mM Pi, as these two Pi concentrations have revealed a clear difference in the colonization frequency.

Transgenic hairy roots were generated and harvested after 28–30 days. Irrespective of colonization success, histochemical GUS reaction revealed a similar blue staining pattern in arbuscule-containing cells from differently Pi-treated roots (Figure 4a, 4c). Co-localization of GUS activity and AM fungal structures by overlay with acid fuchsine staining showed the presence of GUS exclusively in the arbuscule-containing cells (Figure 4e). Epidermal and outer cortical cells, even if crossed by fungal coils, did not show any GUS activity. The histochemical results were in good agreement with the expression pattern previously reported in laser-dissected cells (Guether *et al.*, 2009).

Surprisingly, both in mycorrhiza and non-mycorrhizal hairy roots, GUS activity was also detectable in lateral root primordia as well as in root meristems, independently of Pi concentration (Figure 4b, d, f, g, i, l). Weaker labeling was also present in the central cylinder (Figure 4h, m).

To validate these expression patterns we compared the *LjMAMI* transcript levels of root tips with those from the whole root system. Coherently with the GUS staining, a low but constitutive and Pi-independent expression of *LjMAMI* was found specifically in the root tips of seedlings grown for 28 days at 20 μM or 2 mM Pi, in the absence of the fungus (Figure 5). As expected, *LjMAMI* transcripts were extremely low in the whole roots.

LjMAMI is localized in the nuclei of arbusculated cells

In order to identify the subcellular localization of LjMAMI, its coding region was fused with the 3′ end of the *eGFP*

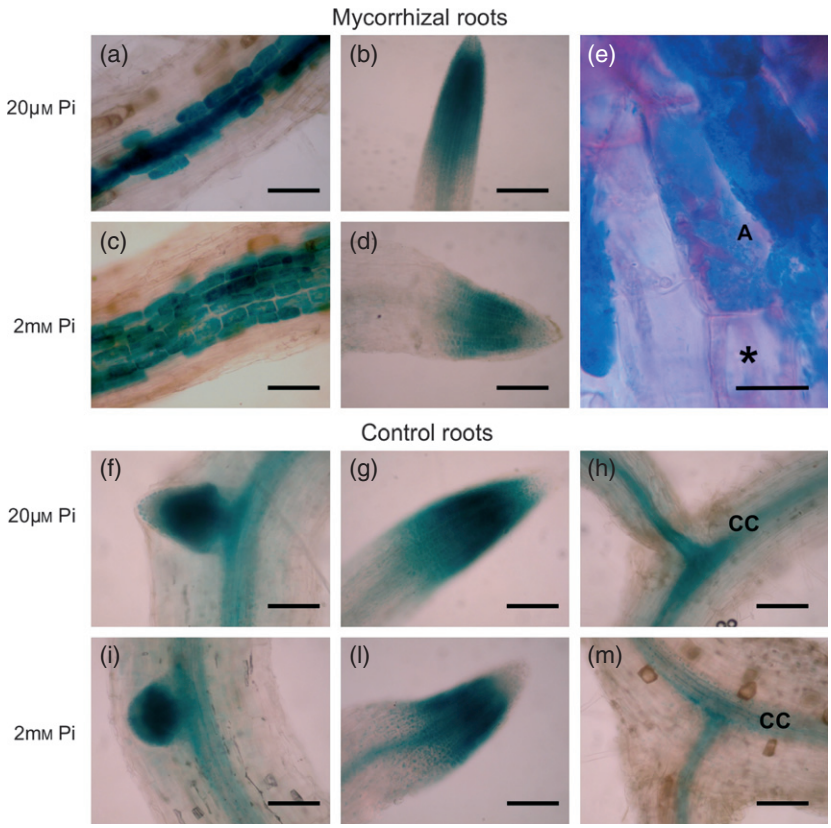

Figure 4. Histochemical GUS staining of *Lotus japonicus* roots expressing pLjMAMI:GUS in the presence and in the absence of *Gigaspora margarita* at two different phosphate (Pi) concentrations.
Mycorrhizal roots grown on 20 μM Pi (a, b, e) or 2 mM Pi (c, d). A vivid blue staining is detected in the arbusculated cells of the cortex (a, c) and in the root meristems (b, d), independent of Pi concentration. (e) Co-localization of GUS activity and acid fuchsine assay: the GUS staining is limited to the arbuscule-containing cells (A), whereas no reaction is detectable in cells that host hyphal coils (*). Control roots treated with 20 μM Pi (f, g, h) and 2 mM Pi (i, l, m) reveal GUS activity in lateral root primordia (f, i) and in the root meristems (g, l). A weaker labeling was also present in the central cylinder (CC; h, m). pLjMAMI:GUS expression is independent of Pi concentration. Scale bars:500 μm (a–d, f–m); 40 μm (e).

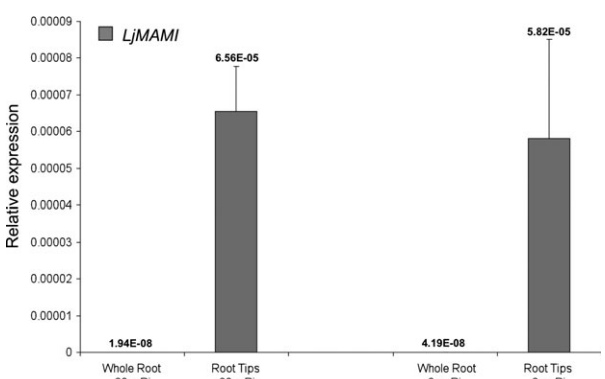

Figure 5. Quantification of *LjMAMI* expression levels in the whole root system and in root tips at 20 μM phosphate (Pi) or 2 mM Pi in the absence of the fungus.
The expression of *LjMAMI* was barely detectable in both whole-root samples. On the contrary, transcripts were found specifically in the root tips, in a Pi-independent manner. Values shown are the means of six replicates, with SEs indicated by the bars.

reporter gene. This chimera was expressed constitutively under the control of the cauliflower mosaic virus (CaMV) *35S* promoter. The GFP fluorescence of protoplasts expressing the chimeric p*35S::eGFP::LjMAMI* was analyzed by confocal microscopy.

In control Arabidopsis and *Nicotiana tabacum* (tobacco) protoplasts, eGFP fluorescence was uniformly extended to the whole cytoplasm (Figure 6—ac and Figure S1a–c), whereas in protoplasts expressing the eGFP-LjMAMI protein the signal was detected in the nucleus (Figure 6–d,f and Figure S1d–f). Interestingly, in Arabidopsis protoplasts the nuclear signal frequently showed a punctuated distribution (Figure 6g). This pattern may be the result of a peculiar subnuclear localization usually associated with transcriptional activity (Lamond and Spector, 2003; Sutherland and Bickmore, 2009).

Consistent with its putative role as a transcription factor, these results indicate that LjMAMI is a nuclear protein.

During previous transcriptome analysis the *LjMAMI* gene was found to be an AM-responsive gene induced in mycorrhizal roots, but limited to arbusculated cells (Guether *et al.*, 2009). To better investigate the *in vivo* intracellular localization of the LjMAMI protein, we generated transgenic roots expressing the eGFP-LjMAMI fusion protein under the control of the native promoter. An expression vector containing the *pLjMAMI:eGFP:LjMAMI* construct was obtained by fusing a genomic DNA fragment containing the 1.5–kb fragment upstream of the *LjMAMI* gene to the *GFP* gene and the coding sequence of *LjMAMI*. Composite plants were inoculated with *Gigaspora margarita*, and after 28–30 days six independent eGFP-expressing root lines were analyzed. In non-colonized cortical cells, GFP fluorescence was never observed (Figure 7—a,c). The GFP signal was detected only in the nuclei of arbusculated

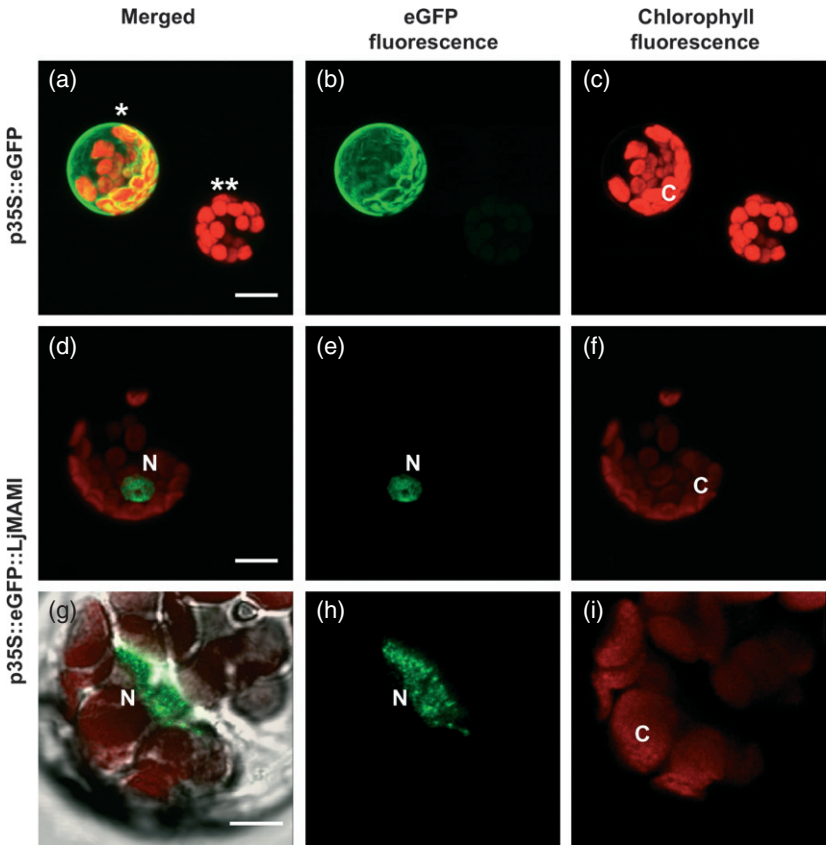

Figure 6. Transient *p35S::eGFP::LjMAMI* expression in Arabidopsis mesophyll protoplasts.
(a–c) Cytosolic eGFP fluorescence is homogenously present inside the control protoplast, whereas the red chlorophyll autofluorescence in the chloroplasts (C) is present in both the transformed (left, *) and non-transformed protoplast (right, **).
(d–f) By contrast, the chimeric eGFP-LjMAMI protein localizes exclusively to the nucleus (N).
(g–i) A higher magnification shows the speckled pattern of eGFP-LjMAMI fluorescence inside the nucleoplasm. Scale bars:10 μm (a–f); 5 μm (g–i).

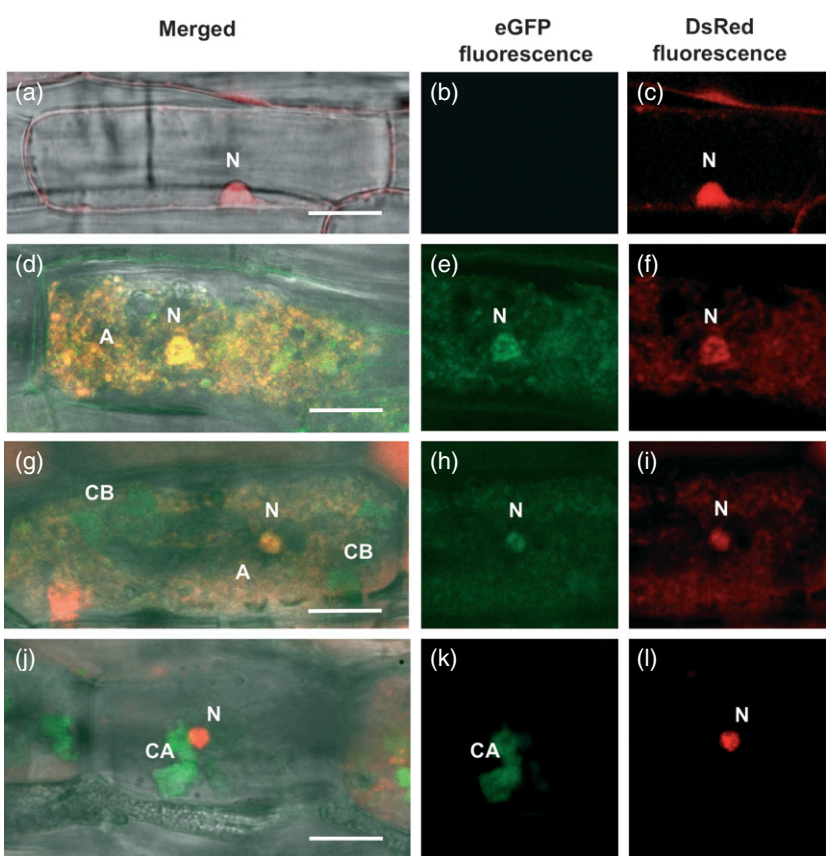

Figure 7. Localization of LjMAMI in mycorrhizal roots. Confocal microscopy images showing the *pLjMAMI:eGFP:LjMAMI* expression in *Lotus japonicus* roots colonized by *Gigaspora margarita*.
(a–c) Non-colonized cortical cell displaying the nucleus (N) in the typical peripheric position and no LjMAMI expression.
(d–f) Cortical cell containing an active arbuscule (A). The nucleus (N), in the characteristic central position, is marked by a strong eGFP-LjMAMI signal, giving a bright-yellow color in the overlay with DsRed.
(g–i) Cortical cell containing an older arbuscule, as indicated by the presence of autofluorescent collapsed branches (CB). A weak eGFP-LjMAMI signal labels the nucleus (N), as confirmed by the orange color in the overlay.
(j–l) Cortical cell with a completely collapsed arbuscule (CA), where no eGFP-LjMAMI fluorescence is detectable in the nucleus (N). Scale bars:20 μm.

cells, and the signal was particularly strong in the cells containing fully developed arbuscules (Figure 7—d,f). By contrast, as soon as the fungal branches started to collapse, initiating arbuscule senescence, the GFP signal became weaker (Figure 7–g,i), and completely disappeared when the arbuscules were entirely collapsed (Figure 7–j,l).

The GFP signal was not detected in root meristems. This discrepancy with GUS staining and qRT-PCR might be the result of lower protein expression in this root region, combined with a higher background for GFP fluorescence and/or rapid protein turnover (de Ruijter *et al.*, 2003).

These data reveal that the LjMAMI protein expression in the nucleus of the cortical cells is associated with the presence of the arbuscule, and is correlated with the arbuscule developmental cycle.

The expression profile of LjPT4, a mycorrhizal functional marker, overlaps with that of LjMAMI, but is dependent on Pi levels

To investigate the temporal expression pattern of *LjMAMI*, we evaluated the levels of *LjMAMI* mRNA by qRT-PCR on roots inoculated with *Gigaspora margarita* at different stages of the colonization process: 3, 6, 9, 14, 21, 28, 35 and 42 days post-inoculation (dpi) (Figure 8a). We also compared the expression levels of *LjMAMI* with *LjPT4* (Guether *et al.*, 2009), which is the Lotus homolog of MtPT4, considered a marker of active arbusculated cells in Medicago (Javot *et al.*, 2007). We found a strong correlation in the expression levels of the two genes (Figure 8a). In particular, *LjMAMI* expression was already detectable at 6 dpi, when the first arbuscules were developed, and reached the highest level at 28 dpi, corresponding to the highest number of arbuscules in our model system (Guether *et al.*, 2009). The last time-course data reveal decreasing *LjMAMI* expression values, correlated with the onset of arbuscule senescence.

As *LjMAMI* was constitutively expressed in a Pi-independent manner in root tips (Figure 5), we wanted to verify whether *LjPT4* displayed a similar regulation. Interestingly, and in contrast with Harrison *et al.* (2002) and Javot *et al.* (2007), *LjPT4* transcripts were also detectable in the root tips, even in the absence of the fungus. However, unlike *LjMAMI* its expression appeared to be dependent on Pi starvation (Figure 8b).

Taken as a whole, these experiments showed that *LjMAMI* expression mirrors the arbuscule development as well as *LjPT4*, and that both genes are regulated in a similar manner during the whole mycorrhization process. Moreover, they are both induced in root tips: *LjMAMI* constitutively and *LjPT4* in a Pi-starvation dependent manner.

Effect *of LjMAMI* downregulation and overexpression on root morphology and mycorrhization

To better investigate the role of LjMAMI we generated transgenic hairy roots containing a *LjMAMI* RNAi construct

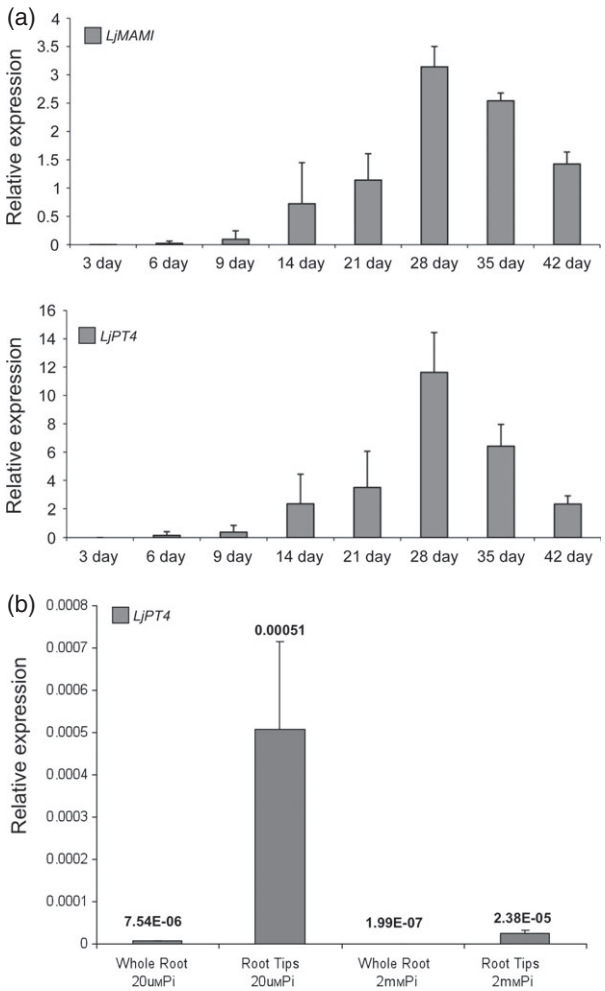

Figure 8. *LjMAMI* and *LjPT4* expression analyses.
(a) The relative expression of *LjMAMI* and *LjPT4* was evaluated in qRT-PCR during a time-course experiment performed on *Lotus japonicus* roots colonized by *Gigaspora margarita*, from 3 to 42 days post-inoculation (dpi). Values are shown as the means of three replicates with SDs.
(b) Quantification of *LjPT4* expression levels by qRT-PCR in the whole root system and in root tips at two different Pi concentrations, 20 μM and 2 mM, in the absence of the fungus. The expression of *LjPT4* was detectable in the root tips and was found to be Pi dependent, reaching the maximum level at 20 μM Pi. Values are the means of three replicates, with SEs indicated by the bars.

or overexpressing *LjMAMI* under the control of a *35S* promoter (see Experimental procedures). A segment at the 3′ end of *LjMAMI* was chosen as the target for downregulation, as this region was extremely divergent from any other available MYB TF sequences of *Lotus* or *Medicago*.

We selected several transgenic hairy root lines that expressed the RNAi marker gene after several days of subculture in Petri dishes supplemented with antibiotics. These roots were then transferred to a medium containing 20 μM Pi, either in the absence or in the presence of the fungus *Glomus intraradices*, currently named *Rhizophagus irregularis*.

Three RNAi lines were obtained and named MAMIi–2, MAMIi–4 and MAMIi–12. The downregulation of *LjMAMI* was verified by qRT-PCR on a region of the *LjMAMI* mRNA that was not used to build the RNAi construct. As *LjMAMI* expression in the whole root system of wild-type non-mycorrhizal plants was already extremely low (Figure 5), we assessed the *LjMAMI* downregulation on mycorrhizal root samples of each line. We detected a strong reduction of *LjMAMI* levels in MAMIi–4 and MAMIi–12 (Figure 9a). In contrast, the downregulation was unsuccessful for the MAMIi–2 line.

In the absence of the fungus, RNAi lines MAMIi–4 and MAMIi–12 grew very slowly and showed a strong reduction in root branching compared with the controls (Figure 9b panel MYC–). The root morphology of RNAi lines was quantified by calculating the total root length (cm) and the number of lateral roots per cm of root, which corresponded to branching points (forks). Quantitative values confirmed a significant difference between control and RNAi roots for both parameters (Figure 9c). The line MAMIi–2, for which the silencing was unsuccessful, presented a root phenotype similar to control roots (Figure 9b, panel MYC–).

All the RNAi lines and their controls were inoculated with *Glomus intraradices*, and after 5–6 weeks their mycorrhization levels and root development were assessed. Surprisingly, in the presence of the AM fungus, root proliferation and emission of lateral roots were enhanced in all cases, especially in the line MAMIi–4 (Figure 9b, panel MYC+). The fungal colonization was diffuse in all lines analyzed, with good mycorrhization parameters (Table S1). Evident differences in the morphology of intracellular hyphae and arbuscules were not detectable in comparison with the control (Figure 10a and c).

The same construct was also used to generate composite plants, where only transformed roots identified by Ds–RED fluorescence under a stereomicroscope were maintained in the root apparatus. Here, again, a reduction of *LjMAMI* expression was detected by qRT-PCR in the transformed roots colonized by *Gigaspora margarita* (Figure S2), which

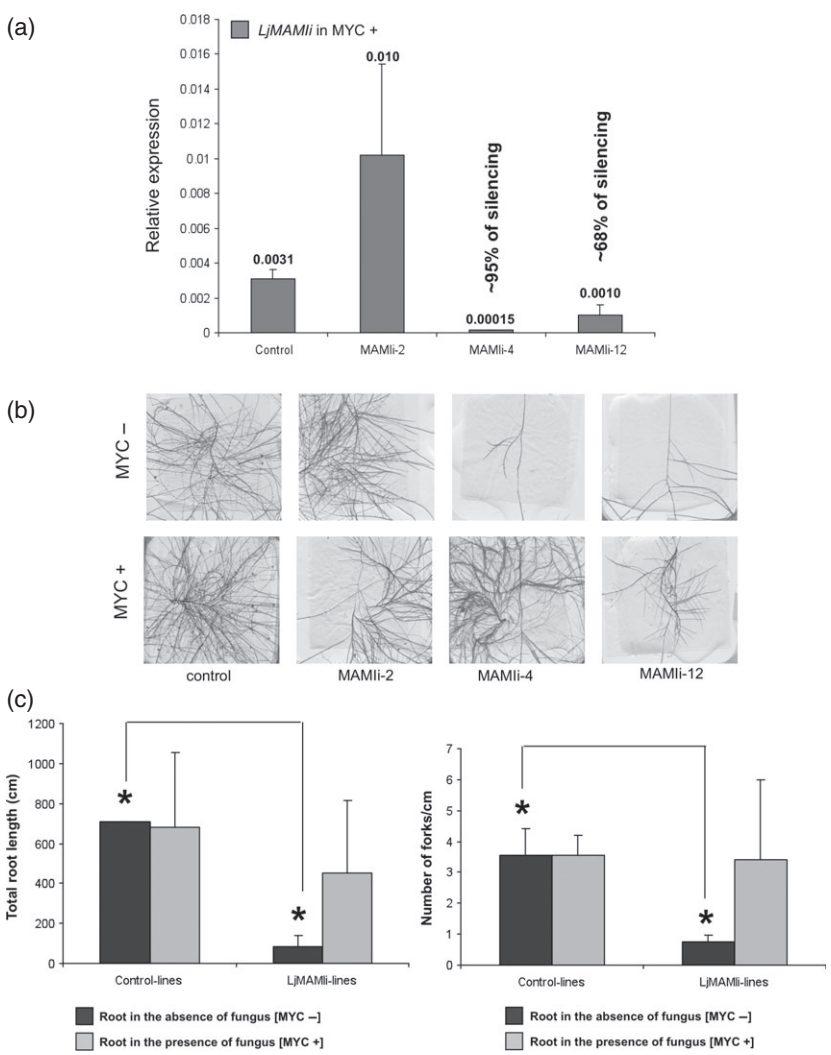

Figure 9. Effects of the downregulation of *LjMAMI* in the RNAi hairy root explants of *Lotus japonicus*.
(a) Validation of downregulation in lines developed in the presence of the fungus through qRT-PCR for the *LjMAMI* gene. A strong reduction in the expression level is revealed only in lines MAMIi–4 and MAMIi–12. The means of two replicates are shown with SE bars.
(b) Root phenotype of control and downregulated lines in the absence (MYC–) and in the presence (MYC+) of the fungus.
(c) Root measurements in the absence of the fungus (dark bars), both total root length (cm) and the number of laterals per root cm, are negatively affected ($P < 0.05$), whereas in the presence of the fungus (light bars) no significant differences are detectable. Two technical replicates of three independent lines were used in each case.

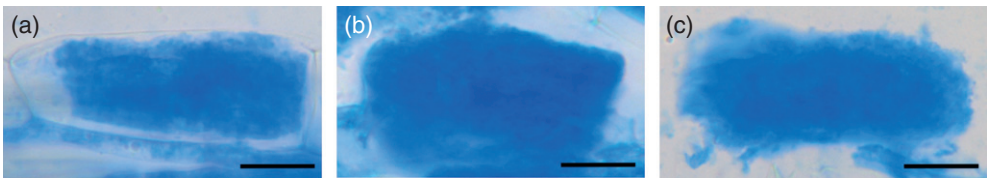

Figure 10. Mycorrhizal phenotype in downregulated, overexpressed and control lines.
No phenotypic differences in the morphology of arbuscules were detectable in LjMAMI RNAi (a) and in the LjMAMI overexpressed (b) lines when compared with control lines (c). Scale bars:40 μm.

showed root architecture comparable with the control plants (Figure S3b). Similarly to the RNAi hairy root lines (Figure 9b, panel MYC–), in the non-mycorrhizal samples an important root growth inhibition was detected (Figure S3a).

Three independent *LjMAMI* overexpressing lines (MYB-AMIoe–7, MYB-AMIoe–10 and MYB-AMIoe–19) were molecularly and morphologically analyzed. In all cases *LjMAMI* expression levels were far higher in comparison with control lines, even in the absence of the fungus, particularly for MYB-AMIoe–7 (Figure 11a). All lines showed lateral root growth and branching similar to control lines in the presence or absence of the fungus, as quantified by the total root length and the number of laterals per cm of root in the overexpressing lines versus control roots (Figure 11b and c). No morphological changes in the arbuscule phenotype (Figure 10b) were detected after mycorrhization.

Taken as a whole, these results showed that altering *LjMAMI* expression had no effect on the establishment of

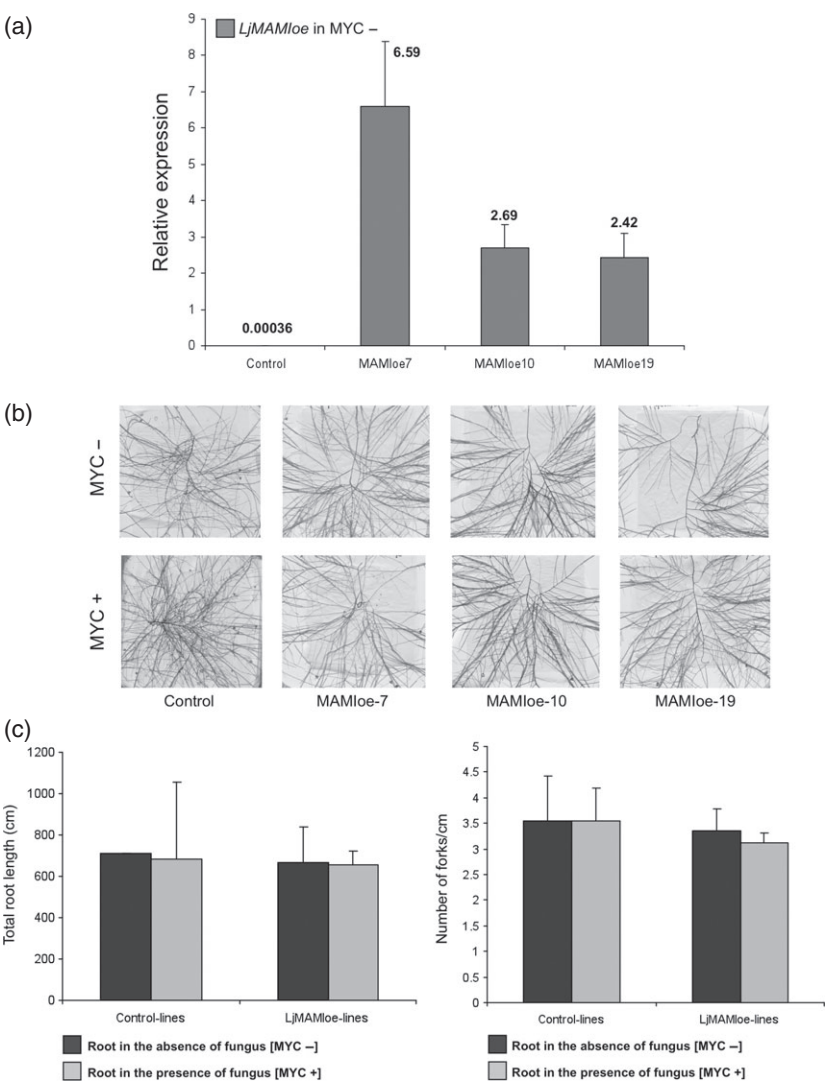

Figure 11. Effects of *LjMAMI* overexpression in hairy root explants of *Lotus japonicus*.
(a) Validation of the overexpression through qRT-PCR for the *LjMAMI* gene in the absence of the fungus. A strong increase in expression is recorded in all the lines, when compared with the control. The means of two replicates are shown with SE bars.
(b) Root phenotype of overexpressing and control lines in the absence (MYC–) and in the presence (MYC+) of the fungus. All the lines display a root architecture comparable with that of the control.
(c) Quantification of the total root length and the number of laterals per root cm did not show any significant difference. Two technical replicates of three independent lines were used in each case.

AM symbiosis or on arbuscule morphology. However, in contrast with the control root phenotype, MAMIi lines showed a strong reduction of root branching and growth in the absence of the fungus. Both the symbiotic fungi used for such experiments seemed to maintain their ability to enhance root branching and growth (Maillet *et al.*, 2011), leading to a partial recovery of the control root phenotype (Figures 9b, b, panel MYC+ and S3b).

DISCUSSION

Root colonization by AM fungi is accompanied by significant cell reorganization, required to accommodate the fungi inside the root cells, and also by important changes in the transcriptomic profiles of the whole plant (e.g. Güimil *et al.*, 2005; Hohnjec *et al.*, 2005; Liu *et al.*, 2007; Guether *et al.*, 2009; Hogekamp *et al.*, 2011; Gaude *et al.*, 2012). The cellular and molecular changes are particularly pronounced in the arbusculated cells, which are considered to be at the heart of symbiosis. The available data support the idea that during AM colonization plants activate an organism-wide reprogramming of their major regulatory networks (Bonfante and Genre, 2010). However, the genetic determinants of such a transcriptional revolution are still mostly unknown.

In this work we describe the biological characterization of a putative MYB TF that is induced by the AM fungi and surprisingly seems to have an effect on root morphogenesis in an AM-independent manner.

The *LjMAMI* gene belongs to a family of TFs involved in Pi starvation and has a nuclear localization

The MYB proteins represent one of the richest families of TFs in plants, and are implicated in various processes such as the regulation of morphogenesis (Ito, 2005), pathogen resistance, cell division, hormonal signaling and response to abiotic stress (Stracke *et al.*, 2001; Du *et al.*, 2009; Feller *et al.*, 2011). R2R3-type MYB TFs are among the best-characterized MYB genes, and may have pleiotropic effects (Romano *et al.*, 2012): some members are involved in Pi starvation signaling, like rice R2R3-MYB OsMYB2P–1 (Dai *et al.*, 2012); others are involved in the regulation of root morphology, like Arabidopsis MYB77 (Shin *et al.*, 2007), which modulates auxin signal transduction and lateral root development.

A detailed phylogenetic analysis revealed that the LjMAMI sequence we identified as highly induced in arbusculated cells (Guether *et al.*, 2009) grouped with the GARP subgroup of MYB-TFs, many of which are required for adaptation to Pi deprivation. The origin of such genes seems to be very ancient, as some members are present in algae such as *Chlamydomonas reinhardtii* and have been maintained in land plants (Paz-Ares *et al.*, 1987; Wykoff *et al.*, 1999; Rubio *et al.*, 2001). One of the best-characterized genes of the group is *AtPHR1*, a MYB-TF essential for the Pi-starvation responses (Nilsson *et al.*, 2007). Like *AtPHR1*, *LjMAMI* expression was seen to be independent of Pi concentration. However, the closest LjMYB-related sequence was an *M. truncatula* MYB-like gene similarly upregulated in arbusculated cells, according to two microarray analyses (Liu *et al.*, 2003; Hogekamp *et al.*, 2011). The sequence data pointed to a clear-cut relationship between *LjMAMI*, the AM symbiosis and the regulation of Pi starvation.

In planta localization assays supported the role of LjMAMI as a transcription regulator, and allowed us to correlate the expression of *LjMAMI* in arbusculated cells with the arbuscule developmental cycle. The localization was also nicely confirmed by *LjMAMI* expression analysis during a time course of the mycorrhization process, as the highest transcript levels were found when arbuscules were more abundant (Guether *et al.*, 2009). Furthermore, in agreement with the hypothesis of a relationship between *LjMAMI* and Pi availability, both *LjPT4* and *LjMAMI* are induced not only in arbusculated cells but also in root tips: the former in a Pi-dependent manner and the latter constitutively. These results suggest unexpected affinities in the transcription profile of root tips and arbusculated cells, which would require further investigation.

When taken together, phylogenetic, localization and expression analyses reveal that the arbuscule-responsive gene *LjMAMI* is a potential nuclear-located TF related to GARP genes, suggesting the hypothesis that it may regulate the expression of some of the major markers of AM functionality, notably related to nutrient assimilation.

The *LjMAMI* gene does not impair the establishment of arbuscular mycorrhiza, but regulates root development

The downregulation and overexpression of *LjMAMI* in hairy roots did not have a direct effect on the mycorrhizal phenotype. In all the lines the AM fungus entered into the root tissues and developed arbuscules with a normal morphology. Mycorrhization levels, in terms of percentage of roots colonized and arbuscules produced, were always comparable with the controls. The results suggest that LjMAMI operates at a different level of the regulatory mycorrhizal network when compared with other currently characterized AM-responsive genes that are involved in arbuscule formation, i.e. *MtPT4* (Javot *et al.*, 2007), vapyrin (Feddermann *et al.*, 2010; Pumplin *et al.*, 2010) and Half-ABC transporters (Zhang *et al.*, 2010). In all these cases RNAi lines revealed clear alterations in their mycorrhizal phenotypes, most often leading to stunted and poorly branched arbuscules. We can therefore conclude that either the RNAi levels we obtained were not strong enough to appreciate this effect or that LjMAMI is not primarily involved in the mechanisms of fungal accommodation. Another hypothesis for the lack of a mycorrhizal phenotype in RNAi roots is that other TFs not yet identified might compensate for the lack of LjMAMI. Indeed, the presence

of a dimerization motif in the gene sequence leads us to hypothesize the involvement of other TFs in the plant response to the fungus that could normally act in synergy with LjMAMI, and that might partially compensate for its downregulation.

On the other hand, histochemical GUS staining revealed a mycorrhizal- and Pi-independent *LjMAMI* expression in specific root tissues. Lateral root primordia and root meristems were the preferential sites of gene induction in the absence of the fungus. RNAi lines also provided evidence for a role of LjMAMI in controlling the root morphology in axenic conditions. In particular, *LjMAMI* downregulation lowers the total root length and the number of lateral roots, suggesting that *LjMAMI* is essential to a correct regulation of root development in the absence of the fungus. Such an effect is at least partially rescued by the presence of the AM fungus. Similarly, it has been reported that a maize mutant lacking lateral roots may recover its original phenotype in the presence of a mycorrhizal fungus (Paszkowski and Boller, 2002).

We concluded that, notwithstanding its strong responsiveness to AM fungi, the downregulation of LjMAMI does not have a direct impact on the mycorrhizal establishment, despite compromising root growth in the absence of the fungus. These findings reveal the presence of a so-far largely unknown network that could link root development with AM symbiosis, perhaps through hormone signaling (Hanlon and Coenen, 2010).

The colonization of the land by plants was aided by their ability to evolve mutualistic AM symbiosis with the ancient Glomeromycota more than 450 Mya. At that time a true root apparatus was probably not yet present or was underdeveloped, leading to complete dependence by the plant on their symbionts for nutrient assimilation (Bonfante and Genre, 2008). It can be speculated that an important step of the plant evolutionary process was the duplication of ancient Pi starvation-responsive genes, which were then, at least in part, recruited for novel functions. By its homology with PHR1, its overexpression in arbusculated cells and its role in root growth, LjMAMI could be an example of such a gene's evolution.

EXPERIMENTAL PROCEDURES

Plant and fungal material

Lotus japonicus seeds were scarified in sulphuric acid, washed three times with sterile water, then incubated for 2–3 min in 1:3 diluted commercial bleach with 1:1000 Triton–X 100 for surface sterilization. After repeated washing with sterile water, the seeds were put on plant agar (6 g l^{-1}) at 4°C for 48 h in dark conditions for vernalization, and then transferred to a climate-controlled chamber at 22°C in dark conditions for germination. Seedlings were then placed onto plant agar square plates at 22°C with a photoperiod of 16–h light and 8–h dark. After 1–2 weeks, seedlings were transferred to pots containing quartz sand. Half of these plants were inoculated with *Gigaspora margarita* spores and grown in climate-controlled rooms at 22°C with the same photoperiod described above. All plants were fertilized twice a week with half-strength Long-Ashton nutrient solution containing 2 μM, 20 μM or 2 mM KH_2PO_4 for the experiment at different Pi concentrations, and 20 μM for the time-course experiment.

The generation of composite plants was performed following the protocol of Stougaard (1995) with *Agrobacterium rhizogenes* 1193 (Stougaard *et al.*, 1987) on *L. japonicus* wild-type seedlings. After the emergence of hairy roots from the root section, seedlings were grown on L medium (Stougaard *et al.*, 1987) supplemented with cefotaxime (0.02% from a 300 mg l^{-1} stock solution) to eliminate *Agrobacterium rhizogenes*. Plants with transformed roots were grown in pots and half of them were inoculated with *Gigaspora margarita*. The selection of transformed roots was performed by analyzing the fluorescence of the reporter gene present in the plasmid employed (DsRED for GUS and subcellular localization experiments, and eGFP for downregulated lines, see Plasmid constructs) using a stereomicroscope (Leica M205 FA, http://www.leica.com).

All plants described above were harvested after 5 weeks. For each experiment, at least three biological replicates were used.

Gigaspora margarita spores were collected from a vernalized pot of inoculated *Trifolium pratense.* Ten spores were used for the inoculation of each plant.

Glomus intraradices, currently *Rhizophagus irregularis* (MUCL 43194), was maintained in the *in vitro* system described by Bécard and Fortin (1988) with *Agrobacterium rhizogenes*-transformed Chicory (*Cichorium intybus* L.) roots in bicompartmental Petri plates, as described by St-Arnaud *et al.* (1996).

RNA isolation, cDNA synthesis and real-time RT-PCR

RNA isolation, cDNA synthesis and qRT-PCR methods have already been described in detail by Guether *et al.* (2009). Prior to qRT-PCR, gene-specific primers for *LjMYB*-rt, *LjUBI*-rt and *LjPT4*-rt were tested on genomic DNA and cDNA. Because RNA extracted from mycorrhizal roots contained plant and fungal material, the specificity of the primer pair was also analyzed by PCR amplification on *Gigaspora margarita* and *Glomus intraradices* genomic DNA. No amplification products were obtained on fungal DNA. The oligonucleotide sequences for all genes studied in this manuscript are listed in Table S2.

5′- and 3′–RACE

Both 5′- and 3′–RACE were performed on total RNA extracted from the mycorrhized roots with the SMART RACE cDNA amplification kit (Clontech, http://www.clontech.com). The PCR product was obtained using the primers *LjMYB*-race-forward/reverse. PCR was performed according to the Clontech protocol using the Advantage 2 PCR enzyme system and 35 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 2 min, with a final extension at 72°C for 10 min. The RACE products were subjected to electrophoresis, cloned in pCRII (TOPO cloning kit; Invitrogen, http://www.invitrogen.com) and analysed by DNA sequencing.

In silico analyses

Sequence analyses were performed with CHROMAS LITE (http://www.technelysium.com.au/chromas_lite.html), BLASTP (available from the National Center for Biotechnology Information, NCBI) and CLUSTALW2. The protein family, domains and functional sites were searched using SCANPROSITE (Bairoch *et al.*, 1997), NETNES (la Cour *et al.*, 2004) and PAIRCOIL 2 (McDonnell *et al.*, 2006).

Phylogenetic analysis was performed using Bayesian inference with MRBAYES 3.2 (Huelsenbeck and Ronquist, 2001) and run through the Cipres Science Gateway (Miller *et al.*, 2010). The Dayhoff substitution model was chosen for the analysis. Four incrementally heated simultaneous Markov chain Monte Carlo algorithms (MCMCs) were run over 2 million generations, until stationarity. Trees were sampled every 200 generations, resulting in an overall sampling of 10 001 trees. The first 2500 trees were discarded as 'burn-in' (25%). For the remaining trees, a majority-rule consensus tree showing all compatible partitions was computed to obtain estimates for Bayesian posterior probabilities (BPPs). The graphic realization of the phylogenetic tree was performed using the iTOL website (Letunic and Bork, 2011).

Plasmid constructs

The coding region of *LjMAMI* was amplified from cDNA using the following primers: *LjMYB*-attB-forward and *LjMYB*-attB-reverse. The amplified fragment was inserted into pDONR221 (Invitrogen) and then recombined, using the Gateway system (Invitrogen), into the binary vector pK2GW7 for overexpression or pK7WGF2,0 for subcellular localization (both Karimi *et al.*, 2002). An *LjMAMI* promoter fragment of 1500 bp was PCR-amplified from genomic DNA using the primers p*LjMYB*-forward and p*LjMYB*-reverse containing *SacI* and *SpeI*, respectively. The promoter fragment was used to replace the CaMV *35S* promoter into pK7WGF2,0 containing the *LjMAMI* coding region. The *LjMAMI* promoter was also fused upstream of the *GUS* gene in the vector pKGWFS7,0. In all three vectors (pK2GW7, pK7WGF2,0 and pKGWFS7,0), the red fluorescent marker DsRED, under the control of the constitutive Arabidopsis Ubiquitin10 promoter (P*Ubq10*) (Limpens *et al.*, 2005), was inserted.

RNAi constructs were created with a 200–bp fragment of *LjMAMI* gene that did not contain the conserved domains. The PCR product, amplified from genomic DNA using the primers *LjMYB*i-forward and *LjMYB*i-reverse was recombined into pK7GWIWG2D (II), which allows hairpin RNA expression under the control of the CaMV *35S* promoter (Karimi *et al.*, 2002). Twelve hairy root lines were obtained and four of them, which expressed the eGFP stably after several rounds of subcultivation, were selected and the gene downregulation was confirmed by qRT-PCR using the primers *LjMYB*i-rt-forward and *LjMYB*i-rt-reverse.

Generation of hairy root explants and mycorrhizal colonization

The generation of hairy roots was performed starting from the protocol described for the generation of composite plants. The transformed roots, selected by visual examination for the DsRED (for overexpressing lines) or the eGFP (for the downregulated lines), were explanted and propagated stably in axenic conditions, in square Petri dishes, containing minimal (M) medium (Bécard and Fortin, 1988) and 20 μM KH_2PO_4. Half of the root apparatus was inoculated with *Glomus intraradices*, leaving the other half to be used as controls.

The *in vitro* system for mycorrhization described in Helber and Requena (2008) was used, with some modifications.

Cellophane membranes (Model 583 Gel Dryer; Bio-Rad, http://www.bio-rad.com) were boiled for 30 min in EDTA-disodium (0.38 g l^{-1}), washed six times with deionized water and autoclaved. Plates of M medium were covered with these membranes and another 20 ml of M medium were then added. Roots and *Glomus intraradices* spores were put on the agar and another cellophane membrane was placed on top in order to isolate the system. This method reduced the formation of root hairs that were not amenable for the fungus, and improved the formation of points of interaction.

Quantification of mycorrhizal colonization

Mycorrhized roots were stained with 0.1% cotton blue in lactic acid and the estimation of mycorrhizal parameters was performed as described by Trouvelot *et al.* (1986) using MYCOCALC (http://www2.dijon.inra.fr/mychintec/Mycocalc-prg/download.html).

Protoplast transformation and confocal analysis

The p*35S*::*eGFP*::*LjMAMI* construct realized in the pK7WGF2,0 vector was introduced into protoplasts of mesophyll cells of tobacco (*N. tabacum*) and Arabidopsis, by polyethylene glycol-mediated transformation, as described previously (Horie *et al.*, 2007; Yoo *et al.*, 2007). Protoplasts were incubated at 20°C in the dark for at least 16 h before microscopy analysis.

Confocal microscopy analyses were performed using a Bio-Rad ViewScan laser scanning microscope (http://www.bio-rad.com/). Excitation and detection wavelengths were at 488 and 515–530 nm, respectively, for GFP, and at 488 and >570 nm, respectively, for chlorophyll. Images were processed using COREL PHOTO-PAINT (Corel Corporation, http://www.corel.com).

Subcellular localization in mycorrhizal roots

The *pLjMAMI*::*eGFP*::*LjMAMI* construct was used to stably transform *L. japonicus* roots via *Agrobacterium*. Root segments, showing DsRED fluorescence and colonized by *Gigaspora margarita*, were excised and fixed in agarose (8%). The agarose block was cut into thin slices (200 μm) using a vibratome and the pieces were put on a slide. Each section was observed using a Leica TCS-SP2 confocal microscope equipped with a long-distance 40 × water-immersion objective (HCX Apo 0.80). GFP was excited with the blue argon ion laser (488 nm) and emitted fluorescence was collected from 500 to 545 nm. DsRed was excited at 488 nm and imaged at 600–700 nm. Under these imaging conditions, the greenish autofluorescence of collapsed hyphae was partially captured by the GFP emission window. Data were collected from a minumum of 15 independently transformed root lines.

Histochemical analysis of root tissue

Lotus japonicus composite plants carrying transformed roots were fertilized with 20 μM and 2mM Pi and inoculated with *Gigaspora margarita*. Control plants were grown in the absence of fungus. Root fragments, showing DsRED fluorescence and extraradical fungal structures, were selected under a stereomicroscope and excised. The root segments were covered with freshly prepared GUS buffer [0.1 M sodium phosphate buffer, pH 7, 0.5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 0.3% Triton X, 0.3% X–Glc]. Samples were incubated at 37°C for 16 h in the dark, washed with distilled water and observed under an optical microscope (Eclipse E400; Nikon, http://www.nikon.com). In the case of roots colonized with *Gigaspora margarita*, AM fungal structures were counterstained with 0.01% (w/v) acid fuchsine in lactoglycerol (lactic acid:glycerol: water, 14:1:1; Kormanik and McGraw, 1982) and observed under an optical microscope (Primo Star; Zeiss, http://corporate.zeiss.com).

Root architeture analysis

Image analysis was performed using WINRHIZO (http://www.regentinstruments.com/products/rhizo/RHIZOTron.html). Scanned images of root plates were processed by this program to calculate the

following parameters: total length of root, number of tips, number of forks and total length of secondary lateral roots.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Subcellular localization of LjMAMI in tobacco protoplasts.

Figure S2. Validation by qRT-PCR of *LjMAMI* gene downregulation in composite plants in the presence of the fungus.

Figure S3. Root morphology of RNAi composite plants in the absence (a) and in the presence (b) of the fungus.

Table S1. Mycorrhization levels for control, MAMIi and MAMIoe lines.

Table S2. List of primers used in this study.

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