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Presence of three mycorrhizal genes in the common ancestor of land plants suggests a key role of mycorrhizas in the colonization of land by plants

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Summary

• The colonization of land by plants fundamentally altered environmental conditions on earth. Plant–mycorrhizal fungus symbiosis likely played a key role in this process by assisting plants to absorb water and nutrients from soil.

• Here, in a diverse set of land plants, we investigated the evolutionary histories and functional conservation of three genes required for mycorrhiza formation in legumes and rice (*Oryza sativa*), *DMI1*, *DMI3* and *IPD3*.

• The genes were isolated from nearly all major plant lineages. Phylogenetic analyses showed that they had been vertically inherited since the origin of land plants. Further, cross-species mutant rescue experiments demonstrated that *DMI3* genes from liverworts and hornworts could rescue *Medicago truncatula dmi3* mutants for mycorrhiza formation. Yeast two-hybrid assays also showed that bryophyte DMI3 proteins could bind to downstream-acting *M. trunculata* IPD3 protein. Finally, molecular evolutionary analyses revealed that these genes were under purifying selection for maintenance of their ancestral functions in all mycorrhizal plant lineages.

• These results indicate that the mycorrhizal genes were present in the common ancestor of land plants, and that their functions were largely conserved during land plant evolution. The evidence presented here strongly suggests that plant–mycorrhizal fungus symbiosis was one of the key processes that contributed to the origin of land flora.

Introduction

Mycorrhizas, dual organs of absorption formed when symbiotic fungi inhabit healthy tissues of most plants (Trappe, 1996), are increasingly recognized to play roles in the cycling of phosphorus, nitrogen, and water in modern terrestrial ecosystems as well as enhancing plant tolerance to abiotic and biotic stresses (Harrison, 2005; Paszkowski, 2006; Parniske, 2008; Smith & Read, 2008). They probably represent a key innovation that helped plants adapt to the harsh terrestrial environment during their initial colonization of land (Pirozynski & Malloch, 1975; Selosse & Le Tacon, 1998; Read *et al.*, 2000; Kottke & Nebel, 2005; Wang & Qiu, 2006; Bonfante & Genre, 2008), as water and nutrient shortages were some of the first challenges plants faced on land (Graham, 1993; Kenrick & Crane, 1997; Gensel & Edwards, 2001). Two lines of evidence have been presented to support this 'mycorrhizal landing' hypothesis. One is the occurrence of mycorrhizas in all major lineages of extant land plants except most mosses (Pirozynski & Malloch, 1975; Harley & Harley, 1987; Trappe, 1987; Boullard, 1988; Read *et al.*, 2000; Brundrett, 2002; Wang & Qiu, 2006; Smith & Read, 2008). The other includes the fossils of glomalean fungi excavated from the Ordovician (Redecker *et al.*, 2000); this group of fungi form arbuscular mycorrhizas with most extant land plants (Pirozynski & Malloch, 1975; Read *et al.*, 2000; Wang & Qiu, 2006). This fossil evidence places the mycorrhiza-forming fungi at the same age as the earliest land plants (Strother *et al.*, 1996; Wellman *et al.*, 2003).

However, three problems may undermine the 'mycorrhizal landing' hypothesis. First, the organs formed between

plant and fungal partners in bryophytes and vascular plants are structurally nonhomologous but functionally analogous. In bryophytes the organs are parts of the haploid gametophyte on the plant (mycorhizoids or mycothalli), whereas in vascular plants they are parts of the diploid sporophyte (Brundrett, 2002; Wang & Qiu, 2006; Ligrone *et al.*, 2007; Bonfante & Genre, 2008). Secondly, despite the report of glomalean fungi from the Ordovician (Redecker *et al.*, 2000), mycorrhizas have not been directly observed in any of the plant fossils from that geological period (Strother *et al.*, 1996; Wellman *et al.*, 2003). The oldest mycorrhizal fossils, found in the underground rhizomes of prevascular plants excavated from the 400-million-yr-old Rhynie Chert of the Early Devonian in Scotland (Remy *et al.*, 1994), are significantly younger than fossils of the earliest land plants (Strother *et al.*, 1996; Wellman *et al.*, 2003). Finally, host shifting by fungi from vascular plants to liverworts has been detected in two recent studies (Russell & Bulman, 2005; Ligrone *et al.*, 2007). This observation has led some to raise the possibility of an alternative 'host-shifting' hypothesis, which postulates that mycorrhizas originated in the common ancestor of vascular plants, and that the presence of these absorption organs in bryophytes was a result of host shifting by fungal symbionts (Selosse, 2005; Ligrone *et al.*, 2007).

To test these hypotheses, we investigated the origin of the genetic machinery that allows plants to engage in symbiotic associations with mycorrhizal fungi. Logically, we focused our effort on the early-diverging lineages of land plants – the paraphyletic bryophytes. Recently, phylogenetic relationships among three bryophyte lineages (liverworts, mosses, and hornworts) and vascular plants have been resolved with several types of molecular data: nucleotide sequences of chloroplast (*atpB* (ATP synthase CF1 beta subunit), *rbcL* (ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit), small subunit (SSU) and large subunit (LSU) rRNA), mitochondrial (*atp1* (ATPase subunit 1) and LSU rRNA), and nuclear (18S rRNA) genes from 192 taxa (Qiu *et al.*, 2007), the presence/absence of 28 mitochondrial group II introns in 16 charophytes and land plants, nucleotide sequences of 67 chloroplast protein genes from 36 taxa (Qiu *et al.*, 2006), and 40 chloroplast genomic structural characters in 18 taxa (Kelch *et al.*, 2004). This well-reconstructed plant phylogeny can serve as a framework with which to investigate the evolutionary history of a gene by comparing the gene phylogeny with the organismal phylogeny. Progress on molecular genetic studies of mycorrhizal development has also made this study possible. Intensive investigation of the nodulation process in legumes over the last 10 yr serendipitously revealed that the rhizobium–plant symbiosis actually evolved from the more ancient mycorrhizal fungus–plant symbiosis, and that the two systems shared many genes in the early stage of microbe–plant communication. Four such symbiotic genes have been identified from *Medicago truncatula* and seven from *Lotus japonicus* (Harrison, 2005; Paszkowski, 2006; Parniske, 2008). Two have been shown to be well conserved for mycorrhiza formation across angiosperms (Chen *et al.*, 2008; Markmann *et al.*, 2008). However, little is known about the genetic mechanism of mycorrhizal symbiosis or even the extent of distribution of these genes in early land plants.

We selected three symbiotic genes for study here because they showed sufficiently high degrees of sequence conservation across the land plants in which they have been investigated. These genes encode proteins that are components of a well-coordinated signal transduction cascade (Parniske, 2008). *DMI1* (Doesn't Make Infections 1), also known as *POLLUX* and *CASTOR* for the two copies in angiosperms, encodes a cation channel located on the nuclear envelope (Ané *et al.*, 2004; Riely *et al.*, 2007). *DMI3* encodes a calcium/calmodulin-dependent protein kinase (CCaMK) that translates nuclear calcium spiking into phosphorylation events (Lévy *et al.*, 2004). The third gene, *IPD3* (Interacting Protein of DMI3) (*CYCLOPS*), encodes a protein that interacts with and is phosphorylated by the DMI3 (Doesn't Make Infections 3) protein in plant nuclei (Messinese *et al.*, 2007; Yano *et al.*, 2008). Mutagenesis and knockout experiments in *M. truncatula, L. japonicus* and *Oryza sativa* have confirmed the requirement of all three genes for arbuscular mycorrhiza formation, and in particular the *dmi3* mutant has a strict nonmycorrhizal (Myc–) phenotype (Morandi *et al.*, 2005; Chen *et al.*, 2008).

Materials and Methods

DNA and RNA isolation, reverse transcriptase–polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE)

The thalli, roots or leaves were collected fresh from the field or from botanical gardens. Detailed information on the provenance of the plant material is included with voucher specimens, which are deposited in the various herbaria listed in Table 1. Total cellular DNAs were extracted with a modified CTAB (hexadecyltrimethylammonium bromide) method (Doyle & Doyle, 1987) or the DNeasy Plant Mini kit (Qiagen). Total cellular RNA was extracted with the RNeasy Plant Mini kit (Qiagen).

To amplify homologs of the gene from diverse land plants, we used the COnsensus-Degenerate Hybrid Oligonucleotide Primer (CODEHOP) (Rose *et al.*, 2003) primer-designing strategy. The target gene fragments were amplified from genomic DNA by PCR, and the products were cloned using the TOPO TA cloning kit (Invitrogen) and sequenced.

The first-strand cDNA was synthesized from 5 μg of RNA with Superscript III reverse transcriptase (Invitrogen)

Table 1 Sequences of the three genes obtained or used in the study[1]

Species	Doesn't Make Infections 1 (*DMI1*)	Doesn't Make Infections 3 (*DMI3*)	Interacting Protein of DMI3 (*IPD3*)	Voucher# (Herbarium)
Liverworts				
Haplomitrium gibbsiae	FJ913205	FJ913229	FJ913199	Engel & von Konrat 28341 (F)
Treubia lacunosa	FJ913206	FJ913230	FJ913200	Engel & von Konrat 28345 (F)
Dumortiera hirsuta	FJ913208	FJ913231	FJ913191	Qiu 06048 (MICH)
Lunularia cruciata		FJ913233 (FJ913254)*		Qiu 01036 (MICH)
Concephalum sp.		FJ913234 (FJ913255)*		Qiu 94096 (IND)
Pellia epiphylla	FJ913207	FJ913232	FJ913196	QW06018 (MICH)
Trichocolea tomentella	FJ913209		FJ913192	Qiu 06038 (MICH)
Mosses				
Takakia lepidozioides	FJ913210 (FJ913225)	FJ913235 (FJ913256)		X-D Li 010 (MICH)
Polytrichum juniperinum	FJ913211	FJ913236		QW06012 (MICH)
Physcomitrella patens	XM_001755593 XM_001758311	AY155462	DS545208	
Fissidens taxifolius		FJ913239	FJ913198	QW06020 (MICH)
Mnium affine		FJ913237		QW06014 (MICH)
Climacium dendroides	FJ913212	FJ913238	FJ913197	QW06019 (MICH)
Hornworts				
Anthoceros agrestis	FJ913213 (FJ913226)			Qiu 99112 (Z)
Phaeoceros laevis	FJ913215	FJ913240		Qiu 06032 (MICH)
Megaceros aenigmaticus	FJ913214	FJ913241	FJ913204	Qiu 06044 (MICH)
Lycophytes				
Lycopodium digitatum	FJ913216	FJ913242	FJ913194	Qiu 08001 (MICH)
Huperzia squarrosa	FJ913217		FJ913195	Qiu 05001 (MICH)
Selaginella moellendorffii	gnllNov06_ contigl32.28 gnllNov06_ contigl187.13	gnllFeb06_ Contigl101.15	gnllFeb06_ Contigl8.14	
Monilophytes				
Botrychium dissectum		FJ913244		Qiu 96214 (IND)
Osmunda regalis		FJ913243		Qiu 02072 (MASS)
Gymnosperms				
Cycas revoluta	FJ913218	FJ913245	FJ913202	Qiu 94051(IND)
Zamia sp.			FJ913203	Qiu 09001 (MICH)
Ginkgo biloba		FJ913246		Qiu 01001 (MASS)
Angiosperms				
Amborella trichopoda		FJ913247 (FJ913257)		Qiu 97123(IND)
Peltandra virginica	FJ913223 (no *DMI1b*)			Qiu 08006 (MICH)
Lilium longiflorum		U24188*		
Smilacina racemosa	FJ913219 (no *DMI1b*)	FJ913248		QW06007 (MICH)
Oryza sativa	NM_001051466 NM_001058400	AC097175.2	EF569223	
Zea mays	DQ403198, DQ403197	DQ403196		
Aquilegia formosa	DT727692+ DT727691, DR940038+ DT940037			
Clematis virginiana	FJ913221 (no *DMI1b*)			Povilus 08002 (MICH)
Vitis vinifera	AM423798, CAO46310	CU459288	CU459357	
Arabidopsis thaliana	NM_124375 (no *DMI1b*)			
Acer sp.		FJ913250		QW06005 (MICH)
Brassica chinensis	FJ913220 (FJ913227)*			Qiu 94122 (IND)
Populus trichocarpa	e_gw1.86.49.1 e_gw1.XIX.2465.1	LG_XI20438946I20441174	LG_III000810	
Euphorbia milii		FJ913249 (FJ913258)*		Qiu 94056 (IND)
Cucurbita pepo	FJ913224*			Qiu 08009 (MICH)
Malus domestica		Z38126*		
Ricinus communis	EEF35956, EEF44668			
Arachis hypogaea		EU395429*		
Cercis canadensis	FJ913222 (FJ913228)*	FJ913251 (FJ913259)*		Qiu 94127 (IND)

Table 1 (*Continued*)

Species	Doesn't Make Infections 1 (*DMI1*)	Doesn't Make Infections 3 (*DMI3*)	Interacting Protein of DMI3 (*IPD3*)	Voucher# (Herbarium)
Lotus japonicus	AB162158, AB162157	AM230792	EF569221	
Medicago truncatula	AY497771, FJ974130	AY502066	EF117279	
Pisum sativum	AJ973194 (no *DMI1b*)	AY502067	EF569222	
Sesbania rostrata		EU622875*		
Lamium sp.		FJ913252 (FJ913260)*		Qiu 95019 (IND)
Coffea arabica		FJ913253 (FJ913261)		Qiu 96113 (IND)
Nicotiana tabacum	EF613118	U70923*, U38446*		
Petunia × *hybrida*	(no *DMI1b*)	EF592572		

[1]All sequences were used in the combined three-gene analyses except those with an asterisk, which were used only in single-gene analyses. The accessions in parentheses contain intron/exon information. The sequences obtained from GenBank are shown in gray.

in a volume of 20 μl with oligo-dT primers. With the first-strand cDNA as a template, the target gene fragments were amplified, and then cloned and sequenced as described above.

To obtain the full-length coding sequence of a gene, we used the RACE method. RNA was treated as suggested in the protocol of the GeneRacer kit (Invitrogen) and reverse-transcribed into first-strand cDNA. With the synthesized cDNA as a template, both 5′- and 3′- cDNA ends of the gene were amplified, cloned, and sequenced.

Phylogenetic and molecular evolutionary analyses

All sequences were aligned at both nucleotide and amino acid levels with ClustalX (Thompson *et al.*, 1997) and Mega 4 (Tamura *et al.*, 2007). Further alignment optimization was carried out manually in McClade (Maddison & Maddison, 2000). A maximum likelihood method of phylogenic analysis using the GTR + I + G model as implemented in RAxML (Stamatakis, 2006) was used to search for the most likely tree and a bootstrap analysis of 100 replicates was also conducted. The analysis was performed at the CIPRES portal (http://www.phylo.org/portal2/). Liverwort sequences were designated as the outgroup as all three genes were absent in the sequenced green algal genomes of *Chlamydomonas reinhardtii*, *Ostreococcus lucimarinus* and *Ostreococcus tauri*.

To detect modes of selection acting on a gene, a branch-site analysis (Zhang *et al.*, 2005) was performed to calculate the ratio (ω) of the nonsynonymous nucleotide substitution rate (d_N) to the synonymous substitution rate (d_S) using PAML (Yang, 2000). Because the topologies of the *DMI1* and *IPD3* gene trees (Supporting Information Figs S1, S3) differed slightly from the land plant phylogeny (Kelch *et al.*, 2004; Qiu *et al.*, 2006, 2007), these two trees were constrained according to the land plant phylogeny so that accurate divergence levels could be estimated. For *DMI3*, the topology obtained in the single-gene analysis (Fig. S2) was used. As mosses, with the exception of *Takakia*, lack mycorrhizas (Boullard, 1988; Read *et al.*, 2000; Wang & Qiu, 2006), and the branch from the ancestor shared by *Takakia* and other mosses to other mosses was exceptionally long, that branch was designated as the foreground whereas the rest of the tree was defined as the background. This analytical strategy was chosen to evaluate whether there was positive selection in that part of the tree and purifying selection in the rest of the tree.

Cross-species mutant rescue experiment and yeast two-hybrid assay

Six bryophytes were selected for cross-species rescue of an *M. truncatula dmi3* mutant: *Dumortiera hirsuta*, *Haplomitrium gibbsiae*, *Megaceros aenigmaticus*, *Polytrichum juniperinum*, *Phaeoceros laevis* and *Treubia lacunosa*. All these bryophytes except *P. juniperinum* are mycorrhizal in natural conditions (Wang & Qiu, 2006). For each bryophyte *DMI3*, the full-length cDNA was first cloned into the pENTR/D vector (Invitrogen), and then transferred into the pK7FWG2-RR binary vector via LR recombination (Invitrogen) to obtain 35S::bryophyte *DMI3* fusion. The vector pK7FWG2-RR was created by cloning the *Aat*II-*Hin*dIII fragment containing the GFP marker under the control of the 35S cauliflower mosaic virus (CaMV) promoter from the Gateway vector pK7FWG2 (Karimi *et al.*, 2002) into a modified Gateway vector, pK7GWIW-G2(II)-*Q10::DsRED* (Limpens *et al.*, 2005). The resulting plasmid DNA was then transformed into *Agrobacterium rhizogenes* MSU440 by electroporation. The *A. rhizogenes* transformants were further cultured on selective media at 30°C for 48 h before use.

Seeds of the *M. truncatula dmi3* mutant (TRV25) were surface-sterilized, synchronized at 4°C for 48 h and germinated by incubation at room temperature overnight. Seedlings of *M. truncatula* TRV25 were transformed by cutting off root tips (3 mm) and then touching the transformed *A. rhizogenes* cells for 5 s with the wounded tips (Boisson-

Dernier *et al.*, 2001). For each *A. rhizogenes* transformant with a bryophyte gene, 40 seedlings were transformed. All treated seedlings were placed on sterile plates containing Farhaeus medium (Catoira *et al.*, 2000) and incubated in a growth chamber (25°C; 16 h day length). After 2 wk, all plants were checked under a fluorescent microscope for red fluorescence (*Ds*RED1), which indicates successful transformation.

Transformed plants were then inoculated with a mycorrhizal fungus, *Glomus intraradices* (IRBV'95; Premier Tech Biotechnologies, Rivière-du-Loup, QC, Canada), either under nonsterile conditions (plants were transferred into pots containing sand and Turface® MVP (Profile Products LLC, Buffalo Grove, IL, USA)) or under sterile conditions (plants were kept inside on sterile plates). In both cases, 100 fungal spores were placed in the root area of each plant. The wild-type *M. truncatula* plants (Jemalong A17) were inoculated with *G. intraradices* as a positive control. Nontransformed *dmi3* mutant plants (TRV25) were inoculated as a negative control. After 6–8 wk, roots were collected for staining. They were cleared in 10% KOH at 90°C for 10 min, acidified in 1% HCl for 5 min, and stained with 0.05% Trypan Blue for 10 min. The stained roots were checked under a light microscope for mycorrhizal infection.

Four bryophytes were included in the yeast two-hybrid assay: *H. gibbsiae*, *M. aenigmaticus*, *P. juniperinum* and *P. laevis*. Their full-length *DMI3* cDNAs were inserted into the vector pBD-Gal4 (Stratagene, La Jolla, California, USA) in a correct reading frame with the DNA-binding motif of the yeast Gal4 protein (Gal4 is a transcription regulator that binds to a UAS (Upstream Activation Sequence) and activates the transcription of reporter genes in yeast two-hybrid). Similarly, a full-length *M. truncatula IPD3* cDNA was inserted into the vector pAD-Gal4. The yeast strain AH109 was sequentially transformed with the fused pBD-Gal4 and pAD-Gal4 plasmids. The *M. truncatula DMI3* cDNA was cloned into the pBD-Gal4 vector and used as a positive control, whereas the empty pBD-Gal4 and pAD-Gal4 vectors were used as negative controls as described previously (Messinese *et al.*, 2007).

All transformed yeast cells were serial-diluted to obtain different concentrations. For each concentration, 4 µl of cells was plated on each of three selection media: LW, lacking leucine and tryptophan (Leu^-, Trp^-); LWH, lacking

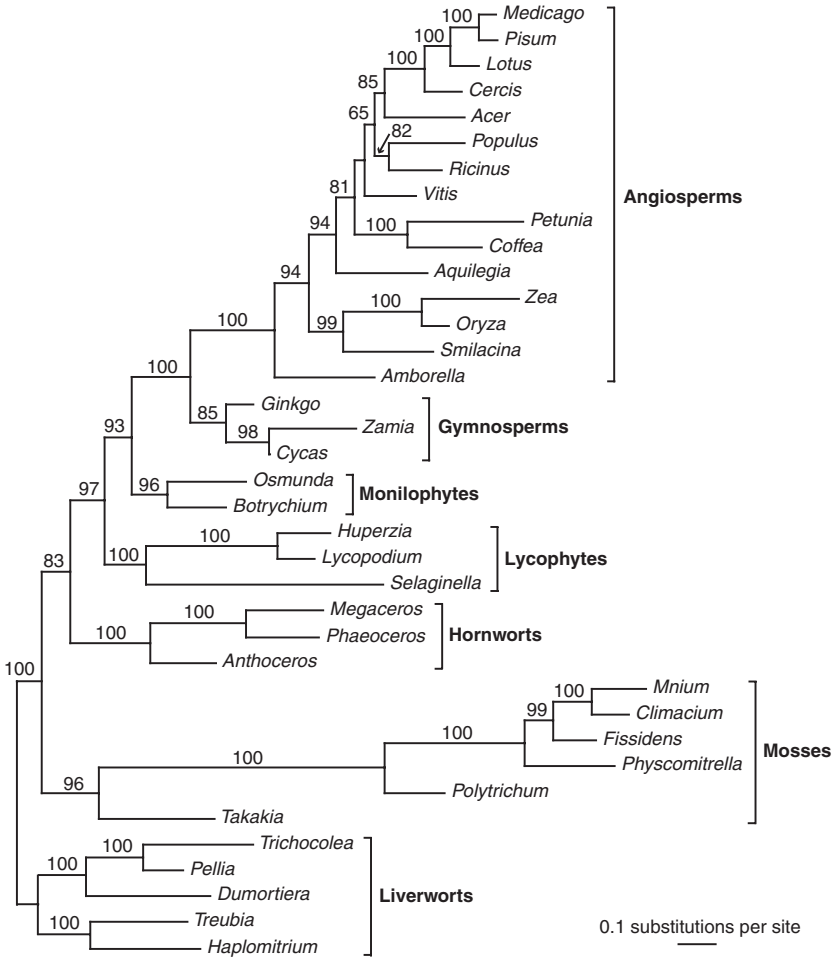

Fig. 1 A maximum likelihood tree obtained from analysis of the combined matrix of three mycorrhizal genes (Doesn't Make Infections 1 (*DMI1*), Doesn't Make Infections 3 (*DMI3*) and Interacting Protein of DMI3 (*IPD3*) of 37 land plants. The numbers above the branches are maximum likelihood bootstrap values. The branch length is proportional to the inferred divergence level. For *DMI1*, *DMI1-1* from *Physcomitrella patens*, *DMI1-x* from *Selaginella moellendorffii* and *DMI1b* from the angiosperms were used to represent the gene in those species.

leucine, tryptophan, and histidine (Leu$^-$, Trp$^-$, His$^-$); and LWHA, lacking leucine, tryptophan, histidine, and adenine (Leu$^-$, Trp$^-$, His$^-$, Ade$^-$). All yeast cells on selection media were cultured at 30°C for 2 d before the results were checked.

Results

Sequences of the three genes were obtained from most of the diverse land plant species targeted (Table 1). Phylogenetic analyses using a maximum likelihood method (Stamatakis, 2006) of these sequences and some from GenBank showed that the three single-gene phylogenies (Figs S1–S3) are generally congruent with the land plant phylogeny reconstructed with both multigene supermatrices and phylogenomic data (Kelch *et al.*, 2004; Qiu *et al.*, 2006, 2007). A combined analysis of the three genes produced a phylogeny (Fig. 1, Fig. S4) in complete agreement with the land plant phylogeny. Moreover, virtually all nodes in the three-gene phylogeny are strongly supported, with bootstrap values > 90%.

For *DMI3* and *IPD3*, only a single copy was isolated from all the species that yielded sequences. These genes probably exist as single-copy genes in the genomes of most, if not all, land plants, as inferred from Southern blots in *M. truncatula* and BLAST searches of sequenced genomes of *Physcomitrella patens*, *Selaginella moellendorffii*, *O. sativa*, *Vitis vinifera* and *Populus trichocarpa* (*Arabidopsis thaliana* lacks mycorrhizas and has neither gene). For *DMI1*, however, two copies were found in *P. patens*, *S. moellendorffii*, and most angiosperms. Their placement in the gene tree suggests that they were derived from recent independent duplications in *Physcomitrella*, lycophytes and angiosperms (Fig. S1). Despite these duplications, the reconstructed

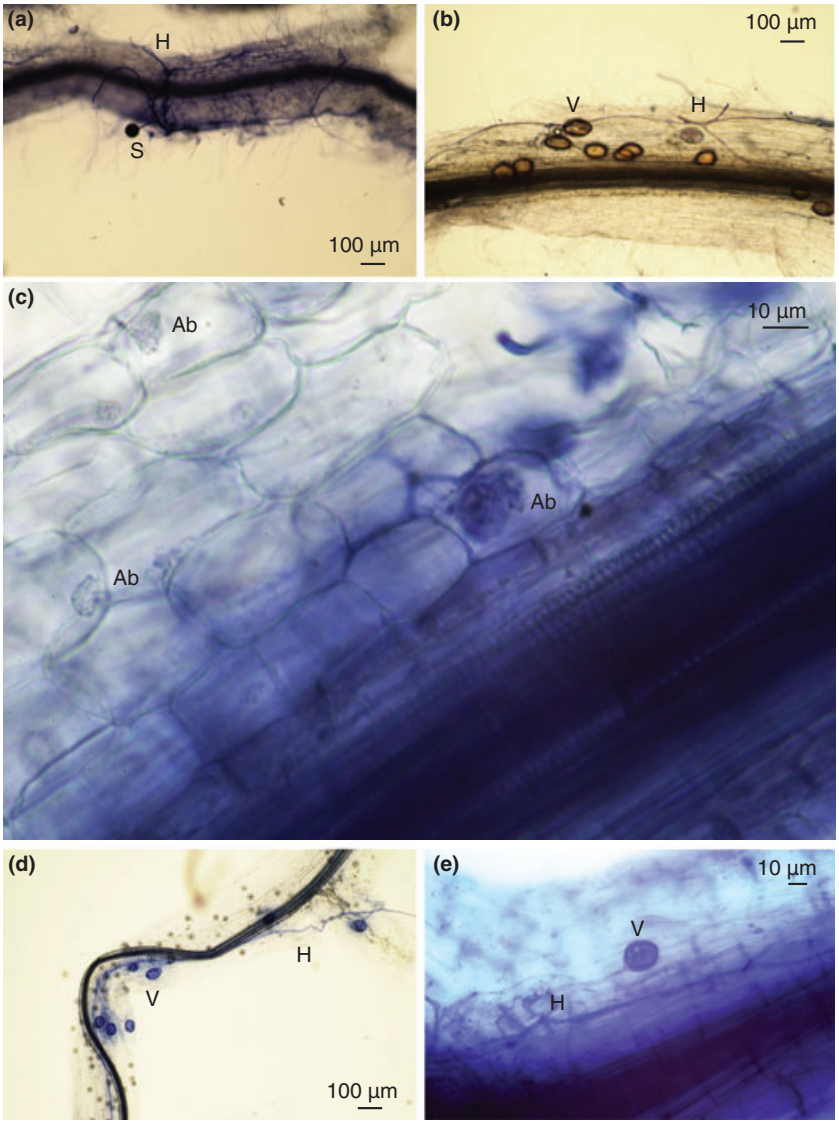

Fig. 2 Recovery of mycorrhizal phenotypes after *Medicago truncatula dmi3* mutant plants were transformed with Doesn't Make Infections 3 (*DMI3*) genes from *Treubia lacunosa* (a–c), *Haplomitrium gibbsiae* (d) (both liverworts) and *Phaeoceros laevis* (e) (hornwort). (a) A root showing a spore (S) and hyphae (H) of *Glomus intraradices*. (b, d, e) A root showing vesicles (V) and hyphae (H). (c) Cortical cells of a root with arbuscules (Ab). All photographs show Trypan Blue staining except (b), where no staining was used.

gene phylogeny suggests that there is an orthologous copy of *DMI1* in most nonflowering land plants.

To evaluate the functional conservation of the three genes, we carried out several analyses. First, *DMI3* was selected for cross-species (bryophytes to *Medicago*) mutant rescue experiments and yeast two-hybrid assays, as the *dmi3* mutant has a strict nonmycorrhizal (Myc–) phenotype (Morandi *et al.*, 2005). These assays were chosen over knock-out experiments in bryophytes because the only well-developed bryophyte model organism, *P. patens*, is like most mosses in lacking mycorrhizas, and the liverwort *Marchantia polymorpha*, which is amenable to some genetic manipulation, has no nonmycorrhizal mutant isolated to date. In the cross-species mutant rescue experiment, full-length *DMI3* sequences of several liverworts, mosses, and hornworts were expressed in *M. truncatula dmi3* mutant plants under the control of a 35S CaMV promoter, which allowed a complete rescue of *dmi3* mutants with the *M. truncatula DMI3* gene. The *DMI3* genes from *H. gibbsiae* and *T. lacunosa* (both liverworts) and *P. laevis* (hornwort) were able to rescue the *M. truncatula* mutant, producing vesicles and arbuscules (Fig. 2, Table S1). The *DMI3* from *P. laevis* was even able to partially rescue the nodulation phenotype of the *dmi3* mutant as a few infection threads (but no nodule primordium) were observed in the presence of compatible rhizobia. However, the gene from *P. juniperinum* (moss) failed to rescue the mutant. This result is consistent with the facts that most mosses lack mycorrhizas (Boullard, 1988; Read *et al.*, 2000; Wang & Qiu, 2006), that their DMI3 proteins failed to interact with the *M. truncatula* IPD3 protein in the yeast two-hybrid assay (Fig. 3), and that their genes show elevated evolutionary rates and may have diverged for new functions (Table 2). For transgenic plants transformed with *DMI3* genes from *D. hirsuta* (liverwort) and *M. aenigmaticus* (hornwort), no recovery of mycorrhizal phenotype was observed, probably because of the small number of transgenic plants generated or insufficient observations made in the study (Table S1). In the yeast two-hybrid assay, DMI3 proteins from *H. gibbsiae*, *M. aenigmaticu* and *P. laevis* could interact with the *M. truncatula* IPD3 protein as the *M. truncatula* DMI3 protein did (the positive control), whereas the DMI3 protein from *P. juniperinum* could not (Fig. 3).

Table 2 The results of the branch-site analyses for selection mode acting on the three mycorrhizal genes in the whole land plant tree excluding the moss branch, and the moss branch

Gene	ω-value for the whole tree excluding the moss branch	ω-value for the moss branch	*P*-value
Doesn't Make Infections 1 (*DMI1*)	0.0545	1.8049	0.1561
Doesn't Make Infections 3 (*DMI3*)	0.0745	2.1763	0.0504*
Interacting Protein of DMI3 (*IPD3*)	0.1065	7.8571	0.0118**

Significance: *, 95% level; **, 99% level; *P*-values are for the test of positive selection acting on the moss branch.

Secondly, branch-site analyses (Zhang *et al.*, 2005) were performed on the three genes to examine the selection mode using PAML (Yang, 2000). Purifying selection was detected for all three genes in much of the tree (ω value < 1), with the sole exception of the branch leading to non-*Takakia* mosses, where the genes were under positive selection (ω value > 1), presumably as a result of functional divergence

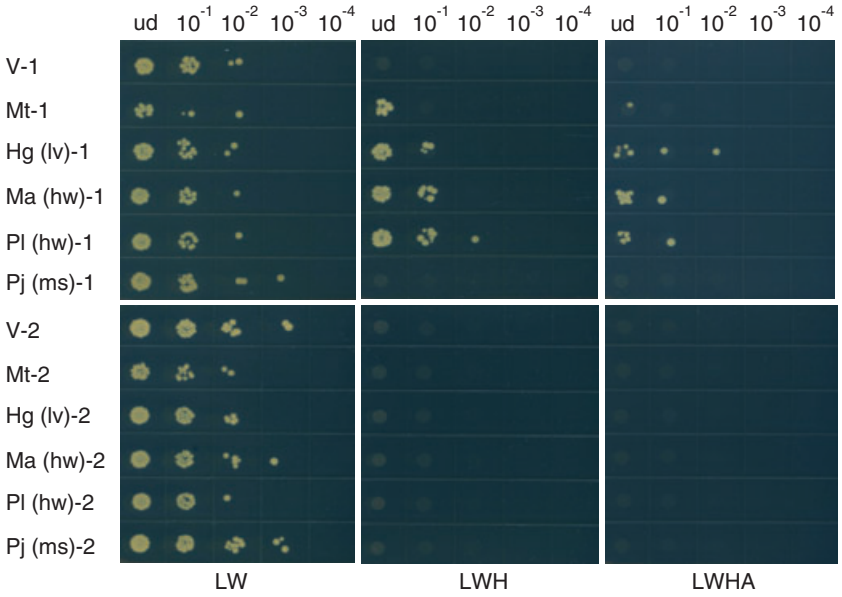

Fig. 3 The yeast two-hybrid assay results. Positive interactions occurred between liverwort (lv) (*Haplomitrium gibbsiae*, Hg)/hornwort (hw) (*Megaceros aenigmaticus*, Ma; *Phaeoceros laevis*, Pl) Doesn't Make Infections 3 (DMI3) proteins and the *Medicago truncatula* (Mt) Interacting Protein of DMI3 (IPD3) protein (lanes Hg-1, Ma-1, and Pl-1), but a negative interaction occurred between the moss (*Polytrichum juniperinum*, Pj) DMI3 protein and the *M. truncatula* IPD3 protein (lane Pj-1). Lane V-1 (no insert in the bait vector pBD-Gal4) and all six treatments in the bottom panel (no *M. truncatula IPD3* insert in the prey vector pAD-Gal4) were negative controls. Mt-1 was a positive control (*M. truncatula DMI3* and *IPD3* in the bait and prey vectors, respectively). Five concentrations of yeast cells were used (ud: undiluted). Selection media were: LW, Leu^- Trp^-; LWH, Leu^- Trp^- His^-; LWHA, Leu^- Trp^- His^- Ade^-.

(Table 2). Purifying selection generally allows maintenance of the ancestral function of a gene during evolution.

Finally, examination of the alignment of the amino acid sequences encoded by the three genes showed that various functional domains identified in the legume proteins (Ané *et al.*, 2004; Lévy *et al.*, 2004; Messinese *et al.*, 2007) were well conserved. The following domains were especially highly conserved between bryophytes and angiosperms: the putative pore and the hinge region in the DMI1 protein, the calmodulin-binding (CaM) domain in the DMI3 protein, and the coiled-coil domain in the IPD3 protein (Fig. S5).

To determine the phylogenetic point at which these three genes originated, we performed BLAST searches in the fully sequenced nuclear genomes of three green algae, *C. reinhardtii*, *O. lucimarinus* and *O. tauri*. None of the three genes was present in the three genomes. Further BLAST searches of GenBank using conserved functional domains of *DMI1* and *IPD3* did not detect any ancestral homolog. *DMI3*, however, is known to be related to calcium-dependent protein kinase (*CDPK*) (Lévy *et al.*, 2004). A comprehensive phylogenetic analysis including most *CDPK* sequences of land plants and green algae and all land plant *DMI3* sequences showed that the land plant *CDPK* and *DMI3* sequences formed two strongly supported clades (Fig. 4, Table S2). Not surprisingly, they were both placed among green algal *CDPK* sequences. While the land plant *CDPK* clade had a short branch, the land plant *DMI3* clade had a rather long branch (the placement of the two lycophytes at the base of the *DMI3* clade is probably an analytical artifact caused by the high degree of divergence between the two *Chlamydomonas CDPK* sequences and the *DMI3* sequences). These results suggest that *DMI3* originated through gene duplication from *CDPK* during green alga evolution and probably also gained a new function after duplication. Because no sequence of either *CDPK* or *DMI3*

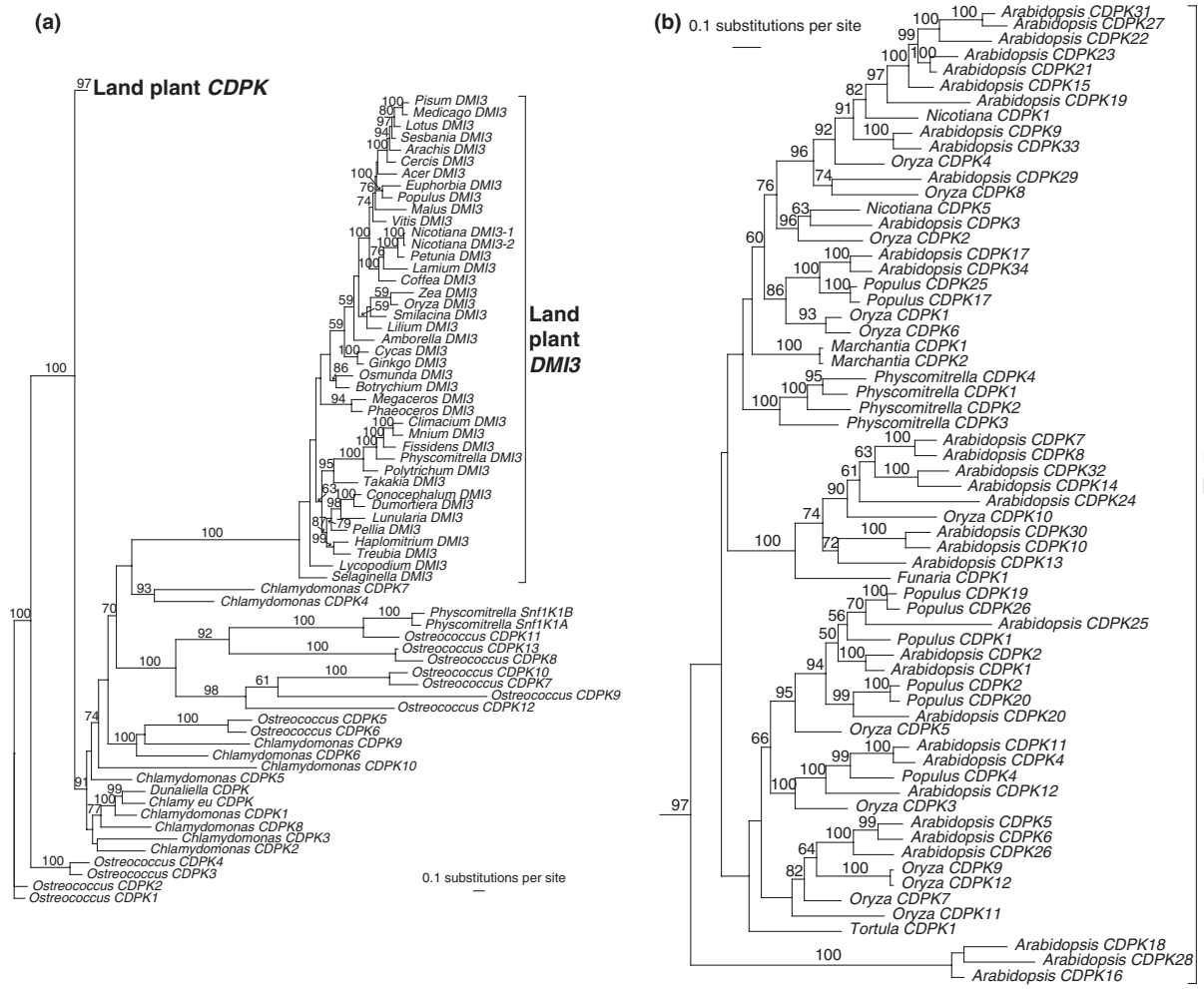

Fig. 4 A maximum likelihood tree derived from green alga and land plant calcium-dependent protein kinase (*CDPK*) sequences and Doesn't Make Infections 3 (*DMI3*) sequences (see Supporting Information Table S2 for sequence information). The numbers above the branches are maximum likelihood bootstrap values. The branch length is proportional to the inferred divergence level.

was available from charophytic algae (our repeated efforts to amplify *DMI3* from *Chara contraria* and *Nitella mucronata* yielded no product), determination of the precise phylogenetic point at which *DMI3* arose in green algae, if it did so, must await future studies. However, the results obtained here are consistent with the idea that the genetic machinery for mycorrhizal symbiosis originated during the colonization of land by plants.

Discussion

The results presented here allow two conclusions to be drawn. The first conclusion is that orthologous copies of the three mycorrhizal genes were present in the common ancestor of land plants, because they are present in nearly all major lineages of extant land plants, including liverworts – the earliest-diverging lineage of land plants (Mishler & Churchill, 1984; Qiu *et al.*, 1998) – and that these genes have been vertically inherited during land plant evolution, as demonstrated by the highly congruent gene and plant phylogenies. The 'host-shifting' hypothesis (Selosse, 2005; Ligrone *et al.*, 2007) requires evidence of horizontal gene transfer, and is thus not supported by the data obtained here. In the original version of the 'host-shifting' hypothesis, there was no elaborate molecular mechanism to explain how host-shifting might have happened. Given that both bryophytes and glomalean fungi were around in the Ordovician (Strother *et al.*, 1996; Redecker *et al.*, 2000; Wellman *et al.*, 2003), one might ask why they did not form symbiotic associations then. One explanation could be that bryophytes did not have the genes and only acquired them later via horizontal transfer from vascular plants, which according to this hypothesis were the first plants with the capacity to form mycorrhizas. A 'reversion hypothesis' was also suggested, which postulates that the common ancestor of land plants had the capability to form mycorrhizas and transmitted it to one lineage (the common ancestor of mosses and vascular plants), whereas other lineages (e.g. non-*Takakia* mosses and liverworts) lost that capability and then acquired it again (e.g. liverworts) (Selosse, 2005). This hypothesis similarly would require regaining of the mycorrhizal genes, which is not supported by the phylogenies of the three mycorrhizal genes reconstructed in this study (Figs S1–S3).

The second conclusion is that the functions of these three genes in controlling the plant–mycorrhizal fungus symbiosis have been largely conserved throughout land plants (with the exception of most mosses), as shown by the results of the cross-species mutant rescue experiments and the yeast two-hybrid assays for *DMI3*, detection of purifying selection acting on all three genes in most parts of the single-gene phylogenies, and the highly conserved functional domains of the three proteins. Lack of any ancient duplication that could be traced back to the beginning of land plant evolution also corroborates this finding, as gene duplication sometimes results in functional diversification (neofunctionalization), as in the case of hemoglobin genes involved in the plant–nitrogen-fixing bacteria symbiosis (Guldner *et al.*, 2004). The consistency of all these results thus unambiguously established the evolutionary homology of symbiotic interactions between gametophytes of liverworts/hornworts and mycorrhizal fungi and those between sporophytes of vascular plants and the fungi.

Over the last 10 yr, morphologically highly similar structures to the arbuscles and vesicles typically seen in mycorrhizas of vascular plants have been found in the thalli or underground organs of diverse liverworts (Carafa *et al.*, 2003; Russell & Bulman, 2005; Duckett *et al.*, 2006; Ligrone *et al.*, 2007) and a hornwort (Schussler, 2000). More recently, a study has demonstrated phosphorus translocation from the fungi *G. intraradices* and *Glomus proliferum* to the host liverwort *Lunularia cruciata* (Fonseca & Berbara, 2008). Given the general functions of mycorrhizas in assisting plants in the uptake of phosphorus, nitrogen, and water, and in enhancing the ability of plants to deal with environmental stresses (Harrison, 2005; Paszkowski, 2006; Parniske, 2008; Smith & Read, 2008), and the barren environment encountered by early land plants (Graham, 1993; Kenrick & Crane, 1997; Gensel & Edwards, 2001), it is reasonable to deduce from the conclusions reached above that plant–mycorrhizal fungus symbiosis played an essential role in the colonization of land by plants.

One might wonder why the genetic machinery underlying the plant–mycorrhizal fungus symbiosis is so conserved across such a wide range of evolutionary divergence, and in particular why there is a lack of duplication for two of the three genes investigated here, as nuclear genes often experience duplications during major evolutionary events. The answer may lie in the fact that arbuscular mycorrhiza symbiosis occurs between *c.* 150 species of glomalean fungi (Morton & Benny, 1990) and a vast majority of the 300 000 species of land plants (Harley & Harley, 1987; Trappe, 1987; Wang & Qiu, 2006; Smith & Read, 2008), and this is the most ancestral form among all mycorrhiza symbioses (Wang & Qiu, 2006). The evolutionary space of the mycorrhizal genes may have been strictly dictated by the small number of glomalean fungal species, which obviously did not diversify much during more than 450 million yr of evolution (Redecker *et al.*, 2000). Repeated formation of partnerships between ascomycetes or basidiomycetes and different plant groups to form other types of mycorrhizas and the much more recent evolution of the interaction between nitrogen-fixing bacteria and some rosid angiosperms were all built on this ancient symbiotic pathway. The genes controlling events downstream of the initial plant–microbe communication signal transduction cascade encoded by the genes studied here can be expected to be less conserved in these other symbiotic systems. The molecular

mechanisms that enabled ascomycetes and basidiomycetes to replace glomalean fungi as symbiotic partners in those plant lineages that adapted to nutrient-poor or nutrient-fast cycling environments (Malloch *et al.*, 1980; Selosse, 2005; Wang & Qiu, 2006; Bidartondo & Duckett, in press) should be of particular interest in future mycorrhiza evolutionary studies.

The origin of land plants was one of the major events in the history of life on earth (Graham, 1993; Kenrick & Crane, 1997; Gensel & Edwards, 2001; Qiu, 2008). The question of how this important evolutionary transition was completed has never been satisfactorily answered. Although it was suggested more than 30 yr ago that plant–mycorrhizal fungus symbiosis might have played an important role in this process (Pirozynski & Malloch, 1975), little progress was made until recently. A significant body of evidence has been gathered over the last 10 yr, which in total seems to paint a fairly consistent picture suggesting that mycorrhizas were intimately involved in the colonization of the land by green plants: the discovery of glomalean fungus and bryophyte fossils in the Ordovician (Strother *et al.*, 1996; Redecker *et al.*, 2000; Wellman *et al.*, 2003), the observation of mycorrhizas in fossils of prevascular plants from the Early Devonian (Remy *et al.*, 1994), the demonstration of mycorrhizal structures in thalli and rhizoids of liverworts and hornworts (Schussler, 2000; Carafa *et al.*, 2003; Russell & Bulman, 2005; Duckett *et al.*, 2006; Ligrone *et al.*, 2007) and nutrient flow through these structures between glomalean fungi and liverworts (Fonseca & Berbara, 2008), and reports of the widespread occurrence of mycorrhizas throughout land plants, with the exception of most mosses (Harley & Harley, 1987; Trappe, 1987; Boullard, 1988; Read *et al.*, 2000; Brundrett, 2002; Wang & Qiu, 2006; Smith & Read, 2008). The molecular evolutionary and functional evidence obtained in this study should now clearly establish the role of mycorrhizas in the origin of land flora.

The results of this study have profound implications in plant biology, ecology, and evolutionary biology. The antiquity of the plant–fungus interaction suggested here, which can be estimated to be at least 480 million yr old given the age of the oldest land plant fossils uncovered to date (Strother *et al.*, 1996), and the widespread distribution of mycorrhizas demonstrated elsewhere (Harley & Harley, 1987; Trappe, 1987; Read *et al.*, 2000; Wang & Qiu, 2006) highlight the necessity of preserving both above- and underground biodiversity in environment conservation efforts. The long history of the plant–mycorrhizal fungus association as suggested here also indicates that there is great potential for the use of mycorrhizas in the development of sustainable agriculture ecosystems (Harrison, 2005; Paszkowski, 2006; Parniske, 2008; Smith & Read, 2008). Finally, the demonstration that fungi directly participated in the initial establishment of the extant terrestrial ecosystems, more precisely biosystems, which operated at an unprecedented level of matter, energy, and information flow (metabolism and reproduction), adds another example to the list of major events in the history of life that were made possible through symbiosis (e.g. the origin of eukaryotes) (Sagan (Margulis) (1967); Maynard Smith & Szathmary, 1995), underscoring the role of symbiosis in the generation and maintenance of biodiversity on earth.

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Supporting Information

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

Fig. S1 A maximum likelihood tree derived from the Doesn't Make Infections 1 (*DMI1*) genes of 33 land plants.

Fig. S2 A maximum likelihood tree derived from the Doesn't Make Infections 3 (*DMI3*) genes of 40 land plants.

Fig. S3 A maximum likelihood tree derived from the Interacting Protein of DMI3 (*IPD3*) genes of 20 land plants.

Fig. S4 A maximum likelihood tree obtained from analysis of the combined matrix of three mycorrhizal genes (Doesn't Make Infections 1 (*DMI1*), Doesn't Make Infections 3 (*DMI3*) and Interacting Protein of DMI3 (*IPD3*)) of 40 land plants.

Fig. S5 Alignment of conserved functional domains in the three mycorrhizal proteins Doesn't Make Infections 1 (*DMI1*) (A), Doesn't Make Infections 3 (*DMI3*) (B), and Interacting Protein of DMI3 (*IPD3*) (C).

Table S1 Results of mycorrhizal formation on *Medicago truncatula* Doesn't Make Infections 3 (*dmi3*) mutant plants transformed with bryophyte orthologs

Table S2 Calcium-dependent protein kinase (*CDPK*) sequences used in this study

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