A Fungal Conserved Gene from the Basidiomycete *Hebeloma cylindrosporum* Is Essential for Efficient Ectomycorrhiza Formation

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Submitted 24 March 2014. Accepted 2 June 2014.

We used *Agrobacterium*-mediated insertional mutagenesis to identify genes in the ectomycorrhizal fungus *Hebeloma cylindrosporum* that are essential for efficient mycorrhiza formation. One of the mutants presented a dramatically reduced ability to form ectomycorrhizas when grown in the presence of *Pinus pinaster*. It failed to form mycorrhizas in the presence of glucose at 0.5 g liter⁻¹, a condition favorable for mycorrhiza formation by the wild-type strain. However, it formed few mycorrhizas when glucose was replaced by fructose or when glucose concentration was increased to 1 g liter⁻¹. Scanning electron microscopy examination of these mycorrhizas revealed that this mutant was unable to differentiate true fungal sheath and Hartig net. Molecular analyses showed that the single-copy disrupting T-DNA was integrated 6,884 bp downstream from the start codon, of an open reading frame potentially encoding a 3,096-amino-acid-long protein. This gene, which we named *HcMycE1*, has orthologs in numerous fungi as well as different other eukaryotic microorganisms. RNAi inactivation of *HcMycE1* in the wild-type strain also led to a mycorrhizal defect, demonstrating that the nonmycorrhizal phenotype of the mutant was due to mutagenic T-DNA integration in *HcMycE1*. In the wild-type strain colonizing *P. pinaster* roots, *HcMycE1* was transiently upregulated before symbiotic structure differentiation. Together with the inability of the mutant to differentiate these structures, this suggests that *HcMycE1* plays a crucial role upstream of the fungal sheath and Hartig net differentiation. This study provides the first characterization of a fungal mutant altered in mycorrhizal ability.

Fungi have evolved different strategies to get the carbon necessary for their growth and development. One of these is a symbiotic association with absorbing roots of plants. This mu-

tualistic association, called mycorrhizal symbiosis, is the most widespread symbiosis on earth (Brachmann and Parniske 2006; Tisserant et al. 2013). In temperate forests, trees form ectomycorrhizas with numerous fungal species, mostly belonging to the phyla Basidiomycota or Ascomycota (Buée et al. 2005; Smith and Read 2008). This association enhances the growth and fitness of trees by improving their mineral nutrition (Alvarez et al. 2009; Landeweert et al. 2001; Pena and Polle 2013) and increasing their ability to withstand adverse conditions (Bojarczuk and Kieliszewska-Rokicka 2010; Colpaert et al. 2011; Sikes et al. 2009). In counterpart, the plant provides photosynthetates necessary for the fungus to complete its life cycle (Nehls 2008; Nehls et al. 2007). Because of its beneficial effect on both plant and fungus, ectomycorrhizal symbiosis plays a key functional role in temperate and boreal forest ecosystems (Courty et al. 2010; Finlay 2008). This symbiosis is remarkable for the conservation of its structural and anatomical features despite the great taxonomic and phylogenetic diversity of the species involved in this association (Smith and Read 2008). It results in the differentiation of ectomycorrhizas which are original mixed fungus–plant organs resulting from a finely tuned interaction between the plant root and the fungus (Plett and Martin 2011). Three major structural components can be distinguished. Extra-radical hyphae radiating in the soil absorb water and nutrients which are translocated to the plant (Casieri et al. 2013). Profusely branched hyphae surround the absorbing root epidermis and form a plectenchyma-like fungal tissue called the sheath, which “insulates” the plant root from the surrounding soil (Ashford et al. 1988). Because of its position, the fungal sheath could control root nutrient uptake and also protect the root against soilborne pathogens (Bücking et al. 2002; Schützendübel and Polle 2002). Finally, the Hartig net, made of fungal hyphae proliferating between root cortical cells, constitutes a large plant–fungus interface where nutrient exchange between the symbiotic partners takes place (Lei and Dexheimer 1988; Peterson and Massicotte 2004).

Differentiation of such integrated structures relies on tightly regulated molecular processes which remain largely to be elucidated (Venkateshwaran et al. 2013). Ectomycorrhiza differentiation is associated with extensive changes in gene transcription (Hacquard et al. 2013; Martin et al. 2008, 2010; Voiblet et al. 2001) and protein biosynthesis (Hilbert et al. 1991). Interestingly, Martin and associates (2008) showed that several genes of the fungus *Laccaria bicolor* encoding small secreted peptides are only expressed or dramatically upregulated during mycorrhizal symbiosis. Plett and associates (2011, 2014) demonstrated that one of them, MiSSP7, plays a crucial

J.-P. Combier and G. Gay contributed equally to this work.

Nucleotide sequence data for *HcMycE1* is available in the *Hebeloma cylindrosporum* Joint Genome Institute database under accession number 23231. The accession number for protein sequence is 23079.

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*The e-Xtra logo stands for “electronic extra” and indicates that six supplementary figures are published online.

role in the molecular cross-talk between this fungus and its symbiotic partner *Populus trichocarpa*. This example validates transcriptome analysis as an experimental strategy to identify essential genes and decipher the molecular basis of ectomycorrhizal symbiosis. However, genes expressed at a low level or moderately regulated can also be essential for symbiosis establishment or functioning. Reverse genetics based on the isolation and characterization of mutants showing a phenotype of interest is an appropriate approach to identify such genes (Nemecek et al. 2006). Insertional mutagenesis has, indeed, been successfully used to obtain nonpathogenic mutants of different fungal species and allowed the identification of several genes essential for pathogenicity (Idnurm et al. 2009; Liu et al. 2013; Maruthachalam et al. 2011).

We previously described the use of insertional mutagenesis to obtain nonmycorrhizal (*myc*⁻) mutants of the ectomycorrhizal fungus *Hebeloma cylindrosporum* (Combiér et al. 2004). Protoplasts of a haploid homokaryotic strain were transformed using polyethylene-glycol-mediated plasmid integration. Out of 1,725 transformants screened for symbiosis defect, 29 turned to be nonmycorrhizal whereas 22 had a dramatically reduced mycorrhizal ability. However, we failed to recover inactivated

genes, because of rearrangements of transforming DNA upon integration in recipient genome (Combiér et al. 2004). In an attempt to circumvent this problem, which has also been reported for pathogenic fungi (Chung et al. 2003; Sanchez et al. 1998), we developed an *Agrobacterium tumefaciens*-mediated transformation procedure (Combiér et al. 2003). The main interest of this method is that transforming T-DNA generally integrates as a single copy in *H. cylindrosporum* genome without rearrangements. This latter feature makes possible the recovery by, for example, thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) of fungal genomic sequences flanking the inserted T-DNA. In this article, we report the characterization of a mycorrhiza-deficient mutant of *H. cylindrosporum* obtained using this method.

RESULTS

Agrobacterium-mediated insertional mutagenesis.

In all, 1,927 T-DNA insertional mutants were obtained using the haploid strain h7 of *H. cylindrosporum*. When cocultivated with the habitual host plant, *Pinus pinaster*, 38 of them showed a dramatically altered mycorrhizal activity because the number

Fig. 1. Schematic drawing of T-DNA insertion in mycorrhiza mutants. **A**, Mutant harboring insertion in the 5' region of a gene. Thin lines: promoter, bold lines: 5' untranslated region. **B**, The mutant 519A2 has a T-DNA insertion in the coding region of the gene *HcMycE1*. White boxes in *HcMycE1* genomic sequence represent exons with roman numerals indicating their length (bp) and lines between boxes are introns. The domain of unknown function (DUF 3638) in which T-DNA integration took place is indicated in gray. Nucleotide regions encoding putative functional domains are indicated below *HcMycE1* genomic sequence. Large arrow indicates T-DNA insertion and orientation by reference to its left and right borders (LB and RB, respectively). The exact position of integration site in *HcMycE1* is indicated above genomic sequence as base pair. The number of base pairs deleted in *HcMycE1* upon T-DNA integration is indicated below the genomic sequence. Likewise, T-DNA sequences deleted upon insertion are indicated above the T-DNA sequence. Mutagenic T-DNA contains an enhanced green fluorescent protein (E-GFP) gene fused to the *Agaricus bisporus* *gpd* promoter and to a *Cauliflower mosaic virus* terminator. A polylinker is also present downstream terminator.

of mycorrhizas per plant was reduced by 90 to 100%. Of these, five never formed mycorrhizas on the Melin-Norkrans medium containing fructose or glucose at 0.5, 1, or 2.5 g liter⁻¹. Southern blot analysis allowed the identification of a single T-DNA insertion locus in the genome of 6 of the 38 mycorrhiza mutants (Supplementary Fig. S1). Another 32 showed unclear Southern blot patterns (not shown) which may result from T-DNA truncation or rearrangement or from multiple T-DNA insertion in the fungal genome. For this reason, they were not studied further. Concerning transformants showing a single T-DNA insertion, TAIL-PCR amplification of genome sequences flanking T-DNA and subsequent locus identification revealed that they all harbored a T-DNA insertion in a gene. In five of them, T-DNA integrated in the 5' end, outside the coding region (Fig. 1A). In mutant 523H1, integration was in the 5' predicted untranslated region (UTR) whereas, in four others, integration was found in the promoters 37, 58, and 59 (twice) bp upstream of the predicted transcription start sites. Although these latter positions look similar, the four corresponding genes did not share any significant sequence homology. In the case of the mutant 519A2, T-DNA integration was located in the coding sequence (Fig. 1B).

Phenotypic characterization of the 519A2 mutant.

Mutant 519A2, which displayed a dramatically reduced mycorrhizal ability, was chosen for further characterizations because it was the only one for which T-DNA integration took place in a coding sequence and for which the observed phenotype, therefore, may not result from changes in gene regulation patterns. This mutant failed to form mycorrhizas when grown in the presence of *P. pinaster* on a Melin-Norkrans medium supplemented with glucose at 0.5 g liter⁻¹, whereas the wild-type strain formed approximately eight mycorrhizas per plant (Fig. 2). The mutant strain, however, formed some scarce mycorrhizas (on average, less than one per plant) in the presence of fructose at 0.5 g liter⁻¹. Both the wild type and the mutant had a higher mycorrhizal activity when co-culture medium contained sugar at 1 g liter⁻¹. Under such conditions, the mutant induced 0.8 and 2.7 mycorrhizas per plant in the presence of fructose and glucose, respectively, whereas the wild-type strain formed 11.3 and 14.3 mycorrhizas on the respective carbohydrate sources.

These preliminary phenotypic characterizations were based on the low magnification stereomicroscope observation of root systems (Supplementary Fig. S2), where mycorrhizas were defined as short roots surrounded by fungal hyphae. Root colonization and anatomy of putative mycorrhizas formed by mutant 519A2 were further studied by scanning electron microscopy (Fig. 3). As suggested from stereomicroscope observations, the mutant was, indeed, able to grow at the surface of *P. pinaster* tap roots (Fig. 3A) and rootlets (Fig. 3B). By comparison with the wild-type strain, which formed a dense hyphal network surrounding mycorrhizas as soon as they emerged from tap root (Fig. 3D, asterisk), mutant hyphal proliferation at a short root surface was very limited (Fig. 3B, double arrowhead). Cross sections in colonized roots showed that the mutant only formed a loose hyphal network (Fig. 3C, asterisk) on the root surface whereas the wild-type strain differentiated a typical sheath made of densely packed hyphae (Fig. 3E, black asterisk). Wild-type mycorrhizas showed a typical Hartig net (Fig. 3E, arrows) that was frequently made of several layers of fungal hyphae. It was not the case with the mutant, which did not form a Hartig net at all (Fig. 3C). From these observations, we concluded that mutant 519A2 was blocked at the presymbiotic stage, before the differentiation of fungal sheath and Hartig net.

In pure culture on the Melin-Norkans agar medium containing different concentrations of glucose or fructose, mutant

519A2 did not show any observable growth defects, either macroscopic (Supplementary Fig. S3) or microscopic, in terms of hyphal morphology (data not shown) compared with the wild-type mycelium. Therefore, the gene affected by mutagenic T-DNA did not seem to play an essential role in vegetative saprotrophic growth.

T-DNA integration.

TAIL-PCR recovered a 700-bp fragment of the genomic sequence flanking the left border (LB) of T-DNA. Search of the corresponding sequence against the *H. cylindrosporum* genome sequence revealed that it corresponded to the 3' end of a putative 9,757-bp putative gene encoding a 3,096-amino-acid protein (protein ID: 23079) of unknown function having a theoretical mass of 351 kDa. T-DNA insertion took place in an exon sequence, 2,873 bp upstream of the stop codon of the gene (Fig. 1B). By reference to the phenotype of the mutant, this gene was named *HcMycE1* (mycorrhiza essential).

Sequence comparison between the wild-type and the mutant strain showed that, upon insertion, 7 bp of genomic sequence was deleted upstream the T-DNA insertion site while no deletion was observed downstream. Likewise, 1,151 and 37 bp were deleted from the T-DNA LB and right borders (RB), respectively (Fig. 1B).

HcMycE1 is a member of a small multigene family with orthologs in numerous fungi and other eukaryotic microorganisms.

BlastP search against the *H. cylindrosporum* protein database indicated that *HcMycE1* protein has three paralogs sharing between 46.7 to 66.7% identity with *HcMycE1*. Searching GenBank and MycoCosm databases using BlastP showed that *HcMycE1* has orthologs in numerous fungi. Among Ascomycota species having orthologs were all members of subphylum Pezizomycotina. Those belonging to Basidiomycota were all members of subphylum Agaromycotina. Homologous sequences were also detected in several other eukaryotic phyla, such as the stramenopiles, alveolata, euglenozoa, or chlorophyta (Fig. 4). No homologous sequences could be identified in prokaryotes, higher plants, and other opisthokont main phyla (including animals) other than the fungi.

Fig. 2. Mycorrhizal activity of the wild-type strain h7 (gray bars) of *Hebeloma cylindrosporum* and of the mutant 519A2 (open bars) associated with *Pinus pinaster*. Mycorrhizal syntheses were performed using the Melin-Norkrans medium containing either glucose or fructose at 0.5 or 1 g liter⁻¹ as sole carbohydrate sources. Mycorrhizal activity was expressed as the average number of mycorrhizal root tips per plant. Data are presented $\pm$ standard error. An asterisk above the mutant bar indicates a statistically significant difference ($P \leq 0.05$) relative to the wild type.

Among fungi, orthologs were identified in several ectomycorrhizal species but not in all of them. Orthologs were also identified in the orchid mycorrhizal *Sebacina vermifera* and in the root endophytic *Piriformospora indica* (Zuccaro et al. 2011). By contrast, we did not identify an ortholog in *Rhizoglyphus irregularis*, the only arbuscular mycorrhizal fungus sequenced so far (Tisserant et al. 2013). Saprotrophic species as *Neurospora crassa*, *Penicillium chrysogenum*, or *Serpula lacrimans* and pathogenic ones such as *Magnaporthe oryzae* or *Botrytis cinerea* also have orthologs. Phylogenetic tree of orthologs identified in representative members of phylum Basidiomycota (Supplementary Fig. S4) did not group species according to their symbiotic or saprotrophic status.

In silico analysis of HcMycE1.

HcMycE1, like all orthologs appearing in Figure 4, has two conserved domains of unknown function (DUF), 3638 and 3645 (Fig. 1B), found in different eukaryotic hypothetical proteins. A motif search using the Interpro protein sequence analysis and classification website allowed the identification

of a putative phospholipase C, phosphatidylinositol-specific X domain (IPR000909), suggesting that HcMycE1 could be a phospholipase C. However, no other functional domain of phospholipases C could be detected. We also detected a P-loop containing nucleoside triphosphate hydrolase domain (IPR027417) which is common to many ATP- and GTP-binding proteins (Saraste et al. 1990). Interestingly, this domain was identified in most fungal orthologs of HcMycE1 (Fig. 4). No correlation could be detected between the presence of this domain and the biological status or the taxonomic position of organisms, a given microorganism frequently having this motif in some paralogs but not in others. By contrast, the phospholipase C, phosphatidylinositol-specific X domain was only detected in HcMycE1.

RNAi inactivation of HcMycE1 dramatically depressed mycorrhizal activity of the wild-type strain.

We first attempted to complement the mutant with the wild-type allele of *HcMycE1* but we obtained no transformant, suggesting that the construct could be lethal or that the transgene

Fig. 3. Scanning electron microscopy observation of *Pinus pinaster* rootlets inoculated with the insertional mutant 519A2 or the original wild-type strain h7 of *Hebeloma cylindrosporum*. Co-cultures were performed on the Melin-Norkrans medium containing fructose at 2.5 g liter⁻¹ as carbon source. Roots were sampled after a 20-day co-culture. **A**, Mutant hyphae proliferated (arrows) at the surface of tap roots without showing increased branching typical of fungal sheath formation. **B**, Mutant hyphae could also grow at the surface of short roots (double arrowheads) but failed to differentiate any sheath. Colonized roots were usually devoid of root hair although a few ones could occasionally be observed (arrow). **C**, Cross section in a short root surrounded by mutant hyphae showed that hyphae proliferating at root surface were loosely packed (asterisk) and did not aggregate and branch to differentiate a fungal sheath. No Hartig net could be observed around host cortical cells (CC). **D**, Young mycorrhizas formed by the wild-type strain generally became dichotomously branched as soon as they emerged from a long root and were embedded in a dense network of fungal hyphae (black asterisk), which stuck to the root (arrow) to form a sheath surrounding the mycorrhiza. **E**, Cross section in a wild-type mycorrhiza showed that it was surrounded by a fungal sheath made of densely packed adherent hyphae (black asterisk). Hyphae proliferating between root cortical cells (CC) formed the Hartig net (arrows), which was frequently pluriseriate. Scale bars: 10 μ m (C and E) and 100 μ m (A, B, and D).

Fig. 4. Phylogenetic tree showing relationship of *Hebeloma cylindrosporum* HcMycE1 to other eukaryote orthologs. The amino acid sequence of HcMycE1 (protein number 23079, arrow) and of representative orthologs retrieved from MycoCosm or GenBank were aligned using MUSCLE. After curing the alignment with GBlock, a phylogenetic analysis was conducted using PhyML. Numbers indicate protein ID. Thick lines delineate bootstrap support above 95% and lower values are given on the branches. Values less than 50% are not mentioned. Black circles indicate proteins having P-loop containing nucleoside triphosphate hydrolase domain.

is too large (9.7 kb plus the antibiotic resistance gene cassette) to be entirely transferred from *Agrobacterium* to the fungus. For this reason, we tried to silence the expression of this gene with an RNAi construct, which involves shorter sequences. The fact that we obtained RNAi transformants suggests that construct size could, indeed, be a limiting factor for *H. cylindrosporum* transformation.

Depending on the transformant strain, RNAi inactivation of *HcMycE1* in the wild-type strain resulted in a 65 to 87% decrease of the gene transcription level (Fig. 5A). *HcMycE1* RNAi inactivation also affected the mycorrhizal activity of transformants (Fig. 5B). RNAi transformants formed between 0.1 and 5.5 mycorrhizas per plant, significantly lower than the 15 mycorrhizas per plant formed, on average, by the wild-type strain. However, no strong correlation existed between *HcMycE1* extinction level and decreased mycorrhiza formation in RNAi transformants. Because RNAi inactivation of *HcMycE1* did not reduce mRNA accumulation of the three paralogs of this gene (Supplementary Fig. S5), it can be concluded that reproduction of the reduced mycorrhizal activity phenotype by RNAi inactivation of *HcMycE1* supports that the symbiosis defect of the 519A2 mutant was, indeed, due to T-DNA insertion at the *HcMycE1* locus.

Fig. 5. RNAi inactivation of *HcMycE1* depressed both transcript level and mycorrhizal activity of a wild-type strain h7 of *Hebeloma cylindrosporum*. **A**, mRNA accumulation in the wild-type strain and in five representative RNAi transformants was studied by quantitative polymerase chain reaction under pure culture conditions on the Melin-Norkrans agar medium. **B**, Under the same culture conditions, *HcMycE1* RNAi dramatically reduced the mycorrhizal activity of all studied transformants. Data are presented $\pm$ standard error. An asterisk above a transformant bar indicates a statistically significant difference ($P \leq 0.05$) relative to the wild type.

Transcriptional regulation of *HcMycE1* in the wild-type strain upon symbiosis establishment.

To determine in which morphogenetic process *HcMycE1* could be involved, its transcription level was quantified using the highly mycorrhizal wild-type haploid strain LPB95 grown in the presence or absence of *Pinus pinaster* as a host plant. Expression was followed over 16 days, the time necessary for fungal mantle and Hartig net differentiation (Tranvan et al. 2000). When grown on the Melin-Norkrans medium supplemented with fructose at 0.5 g liter⁻¹, *HcMycE1* was expressed in the absence of the host plant (Fig. 6A). Transcript abundance increased as a function of time and was approximately two times higher at the end of the culture period compared with time zero. *HcMycE1* expression in mycelia colonizing pine roots was transiently upregulated by approximately fivefold 4 days after inoculation. This transient upregulation corresponded to the time when fungal hyphae proliferated at the surface of pine roots, before fungal sheath and Hartig net differentiation. *HcMycE1* was not upregulated during fungal sheath and Hartig net establishment, which takes place from days four and eight onward, respectively (Tranvan et al. 2000), and was downregulated in mature mycorrhizas. Therefore, *HcMycE1* seemed to be specifically upregulated during presymbiotic stages. This

Fig. 6. *HcMycE1* transcription levels in a wild-type strain of *Hebeloma cylindrosporum* grown on the Melin-Norkrans medium containing fructose at 0.5 g liter⁻¹. The fungus was either grown in pure culture (white bars) or associated with short roots of *Pinus pinaster* seedlings (gray bars). **A**, Transcript accumulation over a 16-day culture period which covers fungal sheath and Hartig net differentiation. **B**, Transcript accumulation at pre-symbiotic stages (i.e., before fungal sheath and Hartig net differentiation). Data are presented $\pm$ standard error. Asterisks indicate a statistically significant difference ($P \leq 0.05$) relative to the corresponding pure culture grown mycelium. Myc = mature mycorrhizas.

was further investigated by quantifying transcript accumulation in free-living mycelia and in hyphae colonizing *P. pinaster* tap roots (Supplementary Fig. S6) over a shorter 4-day period of time and by using a protocol allowing uniform inoculation of the plant root (discussed below). As previously reported (Fig. 6A), transcript accumulation in mycelia obtained in pure culture steadily increased as a function of time (Fig. 6B). By reference to pure culture grown mycelia, *HcMycE1* was transiently upregulated in the presence of host roots 3 days after inoculation, which again suggested a role of *HcMycE1* in pre-symbiotic interaction.

DISCUSSION

Agrobacterium-mediated insertional mutagenesis has been used extensively to identify pathogenicity related genes in several fungi (Huser et al. 2009; Liu et al. 2013; Maruthachalam et al. 2011) but never in symbiotic mycorrhizal species. Of 1,927 *H. cylindrosporum* T-DNA insertional mutants, 38 (i.e., 2%) were dramatically impaired in their mycorrhizal ability. This is nearly half the frequency reported by Combiér and associates (2004), who used plasmid-mediated transformation for the selection of nonmycorrhizal mutants of *H. cylindrosporum*. The lower percentage of mycorrhiza mutants recovered in the present study could be ascribed to the high frequency of single T-DNA insertion (Combiér et al. 2003) as opposed to the frequent multiple integration events observed following plasmid transformation (Combiér et al. 2004; Marmeisse et al. 1992). In the case of *Agrobacterium*-mediated insertional mutants of pathogenic fungi, the frequency of pathogenicity mutants ranged from 0.33% for *Colletotrichum acutatum* (Thalinhas et al. 2008) to 3.9% with *Leptosperia maculans* (Blaise et al. 2007). Thus, *Agrobacterium*-mediated insertional mutagenesis showed the same efficiency in identifying symbiosis and pathogenicity mutants in fungi.

In *Arabidopsis thaliana* and *Oryza sativa* insertional mutants, T-DNA appeared to integrate preferentially in 5' regulatory regions of genes, 3' UTR, and A+T-rich regions (Alonso et al. 2003; Chen et al. 2003; Forsbach et al. 2003; Schneeberger 2005; Szabados et al. 2002; Wang 2008). This was also the case with *M. grisea* (Choi et al. 2007; Li et al. 2007; Meng et al. 2007) and *Cryptococcus neoformans* (Walton et al. 2005) but not with *Colletotrichum higginsianum* (Huser et al. 2009). With five of the six mutants we characterized also having T-DNA insertions in the 5' end of genes, in the promoter 37- to 59-bp upstream transcription start site, *H. cylindrosporum* could represent another example where preferential T-DNA integration occurs in nontranscribed genic regions. The only exception was the 519A2 mutant, which harbored a single T-DNA insertion in an exon. Truncation of the T-DNA LB and RB regions as well as short deletions in the locus where T-DNA integrates are additional features shared between *H. cylindrosporum* and plants (Brunaud et al. 2002; Gheysen et al. 1991; Latham et al. 2006; Thole et al. 2010).

Compared with the wild-type, the 519A2 mutant had a dramatically impaired mycorrhizal ability. RNAi inactivation of *HcMycE1* in the wild-type strain strongly supports the idea that, indeed, the mycorrhizal phenotype of the 519A2 mutant results from mutagenic T-DNA integration in *HcMycE1* and that this gene is essential for ectomycorrhizal symbiosis establishment. Together with the fact that it did not show any growth defect under pure culture conditions, this suggests that the 519A2 mutant was defective in a function specific to the symbiotic process. Scanning electron microscopy observation of colonized rootlets showed that it was unable to differentiate fungal sheath and Hartig net, suggesting that *HcMycE1* plays a crucial role in the early molecular cross-talk between *H. cylin-*

drosporum and *P. pinaster*. In agreement with this hypothesis, *HcMycE1* was found upregulated in the wild-type strain after a 3- to 4-day contact with *P. pinaster* roots (i.e., just before fungal sheath differentiation) (Tranvan et al. 2000).

Because *HcMycE1* encodes a hypothetical protein, its exact function in symbiosis establishment remains elusive. The only informative functional domain we could identify in the protein sequence is an X domain characteristic of phosphatidylinositol-specific phospholipases C (PI-PLC). However, all characterized eukaryotic PI-PLC contain two regions of homology referred to as X and Y domains (Singer et al. 1997). In the case of *HcMycE1*, we failed to detect any Y domain. Moreover, none of the identified homologues had a PI-PLC domain. For these reasons, the probability for *HcMycE1* being a phospholipase C is low. By contrast, the P-loop containing nucleoside triphosphate hydrolase domain identified in *HcMycE1* was also detected in most fungal homologs, suggesting that it could have a conserved function. This motif is common to many ATP- and GTP-binding proteins (Saraste et al. 1990); therefore, it can be hypothesized that *HcMycE1* and its homologues have nucleotide-binding ability. We also identified two additional documented domains of unknown function (DUF 3638 and 3645) in *HcMycE1*. They were also present in all identified orthologs, suggesting that they could also be essential for protein function. This hypothesis is somehow supported by the observation that T-DNA insertion in the mutant genome took place in the DUF 3638 domain located upstream of DUF 3645. As a consequence, *HcMycE1* was transcribed as a short mRNA lacking both DUF domains. This could, at least in part, account for the *myc⁻* phenotype of this mutant. To our knowledge, the regulation of genes encoding proteins containing one or both the DUF domains has never been studied in fungi or in other organisms and the role of these domains remains to be elucidated.

HcMycE1-like proteins appear as being mostly specific in fungi and absent in prokaryotes, land plants, and animals. Orthologs were detected in numerous fungi but not in all of them. Interestingly, a single ortholog was detected in the model ectomycorrhizal *L. bicolor*. The absence of ortholog in some ectomycorrhizal species such as *Amanita muscaria*, *L. amethystina*, and *Tuber melanosporum* is in agreement with the polyphyletic origin of the ectomycorrhizal symbiosis (Hibbett and Matheny 2009; Hibbett et al. 2000). It shows that, although ectomycorrhizal symbiotic structures are highly conserved, molecular mechanisms underlying their differentiation can be variable. The presence of orthologs in pathogenic and saprotrophic fungi indicates that *HcMycE1*-like proteins can have various functions. The fact that most of the microorganisms (fungi, alveolata, euglenozoa, or stramenopiles) having *HcMycE1* orthologs live in close interaction with a living host suggests that, in addition to being essential for mycorrhizal symbiosis establishment, *HcMycE1*-like proteins could also be involved in pathogenic interactions. Noticeably, those belonging to euglenozoa and alveolata as well as *Aspergillus fumigatus* cause important human diseases. Because *HcMycE1*-like proteins were also found in the phagotrophic amoebae *Dictyostelium fasciculatum* and in chlorophyta which do not interact with a host, these proteins could also play other roles. The experimental assessment of this hypothesis in model species which can be genetically manipulated would improve our understanding of the role of these proteins and of their DUF domains.

MATERIALS AND METHODS

Biological materials.

The *H. cylindrosporum* haploid strains h7 and LPB 95 were isolated from single-spore germinations (Debaud and Gay

1987). The insertional mutant 519A2 has been obtained via *Agrobacterium tumefaciens*-mediated transformation of the haploid strain h7 (Combiér et al. 2003). All the fungal strains were maintained on YMG agar medium (Rao and Niederpruem 1969) at $22 \pm 1^\circ\text{C}$ in the dark.

Fungal pure culture and mycorrhizal syntheses.

In vitro ectomycorrhizal syntheses were performed in 140-mm petri dishes according to Gea and associates (1994). Sterile 2-week-old *P. pinaster* seedlings were placed on the surface of a solid modified Melin-Norkrans medium (Norkrans 1949) supplemented with Heller's micronutrients (Gay 1990) and glucose or fructose at different concentrations as a carbon source. To obtain fully differentiated mycorrhizal short roots, inoculation was performed by placing inoculum agar plugs (4 mm in diameter) every 5 mm along seedling tap roots. Each experiment consisted of 10 petri dishes, each containing two plants, so that the average mycorrhiza number per plant was evaluated on 20 plants. After a 3-week co-culture period, root systems were observed under stereomicroscope ($\times 40$) to check for the presence or absence of mycorrhizas, defined as short lateral roots usually dichotomously branched, devoid of root hair, and surrounded by fungal hyphae. For experiments focusing on presymbiotic stages of the interaction, roots were uniformly inoculated by dipping into a mycelial homogenate obtained as follows. Two-week-old mycelia obtained on YMG agar medium were roughly macerated using an Ultra-turrax homogenizer and the hyphal fragments were transferred to YMG liquid medium and grown for 1 week. Maceration followed by cultivation in fresh liquid medium was repeated every 2 days in order to get a fast-growing mycelium. Two thalli obtained in 90-mm petri dishes were then macerated and resuspended in 30 ml of Melin-Norkrans medium supplemented with fructose at 0.5 g liter^{-1} . After a 3-day culture period, mycelia were again resuspended, macerated, and used as inoculum.

Scanning electron microscopy.

At the end of the co-culture period, rootlets were excised under a stereomicroscope and fixed for 2 days at room temperature in 3% glutaraldehyde dissolved in 0.1 M citrate phosphate buffer, pH 7.0. Samples were then dehydrated in a graded

series of acetone solutions and, finally, processed through CO_2 critical point drying. They were then mounted on scanning electron microscopy stubs and coated with gold-palladium alloy. Observation was performed on a Hitachi S800 scanning electron microscope operated at 15 KV.

TAIL-PCR analysis of the integration junctions.

DNA fragments spanning the insertion junction of T-DNA were amplified by TAIL-PCR using the method developed by Liu and associates (1995) and Liu and Wittier (1995) as modified by Combiér and associates (2003). Degenerated primers (AD1, AD2, and AD3) as well as LB- and RB-specific primers were described by Combiér and associates (2003). Amplified products from successive PCR were analyzed by agarose gel electrophoresis. A single PCR product was generally obtained following the third round of PCR. All DNA fragments from the tertiary PCR showing a decrease in length (between the second and third PCR) consistent with primer positions on the T-DNA were assumed to be T-DNA junctions and sequenced.

Upon T-DNA integration in genomes, deletions can occur in genomic sequence as well as T-DNA. Deleted nucleotides were identified by amplifying T-DNA LB and RB junctions using the primers listed in Table 1.

In silico analysis of HcMycE1.

In silico analysis of HcMycE1 and its homologues was based on information available on MycoCosm and on the National Center for Biotechnology Information blastp-associated putative conserved domain identification tool. A search for functional domains was also performed using the Interpro protein sequence analysis and classification website. In case of fungal genomes available on websites other than the Joint Genome Institute, the website has been indicated.

Phylogenetic analyses.

The protein sequences homologous to HcMycE1 retrieved from the proteomes of different eukaryotes were aligned using the MUSCLE software. Phylogenetic uninformative positions were removed using GBlock with low stringency settings. Phylogenetic analysis was conducted using PhyML as implemented on the phylogeny.fr platform (Dereeper et al. 2008).

Table 1. Primer sequences used in this study

Primer	Primer sequence (5'-3')	Use ^a
519RTU	CTCGAGTTCAAGGCGTCTTA	RNAi construct
519RTL	CCAGGCCATAGTCCACAC	RNAi construct
<i>Pp-GH3.16U</i>	CCATTCTTTCTGCTCACC	RNAi construct
<i>Pp-GH3.16L</i>	GAACACAGTATCGCCTCC	RNAi construct
519qPCR2F	CGACTCGAAATTTGGTGCAT	<i>HcMycE1</i> transcript quantification by qPCR
519qPCR2R	GACTCGACCAGAACCGCAT	<i>HcMycE1</i> transcript quantification by qPCR
10316 1F	CGGTCTCACGAATCTCAAGAAG	Transcript quantification of the <i>HcMycE1</i> paralog number 10316
10316 1R	AGTCATCCGAGTCAGCGTGATG	Transcript quantification of the <i>HcMycE1</i> paralog number 10316
26859 1F	TGCTACAGCCTTGGCCGACT	Transcript quantification of the <i>HcMycE1</i> paralog number 26859
26859 1R	CGTCACTAAGACTCCTTGGACT	Transcript quantification of the <i>HcMycE1</i> paralog number 26859
409020 2F	CACTCCGAATGACCCCGTTA	Transcript quantification of the <i>HcMycE1</i> paralog number 409020
409020 2R	ACTGGGTCGCTGTGCAAGGT	Transcript quantification of the <i>HcMycE1</i> paralog number 409020
gpdFH	GAAGAAGCTTTAAGAGGTCCGC	Boarding sequence recovery by TAIL-PCR
gpdU	TAAGAGGTCCGCAAGTAGATTG	Boarding sequence recovery by TAIL-PCR
AM2F	CGTCATCGTACCCCTTGTAGCC	Boarding sequence recovery
AM2R	GAGTGTTCATGGTAATGTCACGGGAGA	Boarding sequence recovery
AV1F	TGAGTTGGTTCCTGGAGAGGTGCG	Boarding sequence recovery
AV1R	AGATTGCGGGCTAAGGTCCAC	Boarding sequence recovery
3UTR2F	GTTTCGCTCATGTGTTGAGCAT	<i>HcMycE1</i> transcript detection by RT-PCR
3UTR3F	ATACCATGGACCACAGCAG	<i>HcMycE1</i> transcript detection by RT-PCR
519AV6F	AACATCATATACCATGGACCA	<i>HcMycE1</i> transcript detection by RT-PCR
EF1 α 2F	CTCATCATCGCCGGTGAACGTG	<i>EF1α</i> transcript quantification by qPCR
EF1 α 2R	CTTGGTGGTGTCCATCTGTGTTGACG	<i>EF1α</i> transcript quantification by qPCR

^a qPCR = quantitative polymerase chain reaction, TAIL = thermal asymmetric interlaced, and RT = reverse transcriptase.

Bootstrap values were calculated from 1,000 trees. Unpublished protein sequences produced by the United States Department of Energy Joint Genome Institute in the framework of the Mycorrhizal Genomics Initiative consortium were used by special authorization of Dr. F. Martin, coordinator of the project.

HcMycE1 RNAi inactivation.

For the RNAi construct, a fragment containing the hygromycin-resistance cassette and the divergent *gpd* promoter of the pBGgHg plasmid (Chen et al. 2000) was amplified using *Pfu* polymerase (Invitrogen) and was cloned into *SacI*-*SacII* restriction sites into the pBluescript (pBS) plasmid (Stratagene). In parallel, a fragment of 679 bp of the *HcMycE1* gene was amplified using 519RTU and 519RTL primers (Table 1) and cloned into a pGEMT plasmid (Promega Corp.). The *HcMycE1* fragment was inserted in the pBS plasmid containing the hygromycin cassette with *NotI*-*SacII* restriction sites. The terminator of the hygromycin B phosphotransferase (*hph*)-encoding gene of *Schizophyllum commune* was amplified from pGHGHT (Schuren and Wessels 1998) and digested with *Bam*HI-*Eco*RI to be inserted in the previous plasmid. The antisense fragment of *HcMycE1* gene was amplified and digested with *NotI*-*Bam*HI and inserted in the plasmid. The linker was a 539-bp-long fragment of the *Pp-GH3.16* gene from *P. pinaster* (GenBank accession number AM042714). It was amplified using *Pp-GH3.16U* and *Pp-GH3.16L* primers (Table 1) and digested with *NotI* to be cloned between sense and antisense fragments. Finally, the entire RNAi cassette was inserted in pPZP plasmid (Hajdukiewicz et al. 1994) by using *SacI*-*KpnI* sites.

RNA extraction and quantitative reverse-transcription PCR.

H. cylindrosporum total RNA from free-living mycelia, inoculated pine roots, or ectomycorrhizas was extracted as described by Bailly and associates (2007). Three independent biological replicates were performed for each test. A DNase I treatment was performed after an additional precipitation of the RNA with 3 M Na acetate and ethanol. cDNAs were synthesized by reverse transcription (RT), using 250 ng of total RNA, 200 U of M-MuLV RevertAid reverse transcriptase (Fermentas), 20 U of RNase inhibitor (Fermentas), and 50 nM random hexamers (Fermentas) and by following the manufacturer's recommendations. cDNAs were amplified by PCR using 4 µl of the cDNA reaction mixture, 2 mM dNTPs (Euromedex), 0.5 U of *Taq* polymerase (Invitrogen), and 0.5 µM each of the gene-specific PCR primer (Table 1). Amplification programs were adapted to each primer set.

RNA gel electrophoresis did not reveal any visible RNA degradation or DNA contamination. Moreover, primers used for real-time quantitative PCR (qRT-PCR) have been designed for having a PCR product overlapping an intron. For this reason, the PCR product obtained from cDNA is expected to be 98 bp long, whereas it would be 144 bp long from genomic DNA. Gel electrophoresis of RT-PCR product only revealed a 98-bp-long product. Finally, q-PCR also resulted in the amplification of a single DNA fragment.

qRT-PCR was performed in triplicate using the LightCycler 480 SYBR green master I kit (Roche) on a Lightcycler 96 real-time PCR system (Roche). All primers used for qRT-PCR are listed in Table 1. Their efficiencies were above 98%. Amplifications were performed using the following program: 95°C for 15 min, followed by 45 cycles of 95°C for 15 s, 60°C for 20 s, and 72°C for 20 s. At the end of the run, melting curves were generated to evaluate the amplification specificity. Expression levels were calculated using the cycle threshold ($2^{-\Delta\Delta CT}$) approach (Livak and Schmittgen 2001), using a constitutive gene (elongation factor 1- α) as a control.

Statistical analysis.

Data were expressed as mean values $\pm$ standard deviation. Statistical analysis of significance was performed using the computer software package XLSTAT (Addinsoft; add-in to Microsoft Excel), which first tests the data for normality and equal variance. Data that met the criteria for parametric tests were analyzed by a Student *t* test. When groups of data failed tests for normality and equal variance, they were analyzed by a Wilcoxon test. A *P* value of less than 0.05 was considered as statistically significant.

ACKNOWLEDGMENTS

J. P. Combiér was supported by a grant from the French Ministry of National Education. Scanning electron microscopy was performed at the Centre Technologique des Microstructures (CTµ), University Lyon 1. qPCR facilities were provided by the DTAMB technical platform, University Lyon 1. The genome sequence data were produced by the U.S. Department of Energy Joint Genome Institute in collaboration with the user community. The work conducted by the U.S. Department of Energy Joint Genome Institute is supported by the Office of Science of the U.S. Department of Energy under contract number DE-AC02-05CH11231. We thank F. Martin and the Mycorrhizal Genomics Initiative consortium for access to unpublished genome data and D. Abrouk for statistical analyses.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- Broad Institute *Botrytis cinerea* datatbases: www.broadinstitute.org/annotation/genome/botrytis_cinerea/MultiHome.html
- Broad Institute *Magnaporthe oryzae* database: www.broadinstitute.org/annotation/fungi/magnaporthe/MultiHome.html
- Broad Institute *Neurospora crassa* database: www.broadinstitute.org/annotation/genome/neurospora/MultiHome.html
- EMBL-EBI Bioinformatics services database: www.ebi.ac.uk/services
- GenBank database: blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins
- Interpro server: www.ebi.ac.uk/interpro/
- Joint Genome Institute *Hebeloma cylindrosporum* genome: genome.jgi.doe.gov/Hebcy2/Hebcy2.home.html
- MycCosm database: genome.jgi.doe.gov/programs/fungi/index.jsf
- National Center for Biotechnology Information: blast.ncbi.nlm.nih.gov/Blast.cgi
- Phylogeny online programs, including Gblocks: www.phylogeny.fr/version2.cgi/index.cgi
- URGI Plant and Fungi data integration databases: urgi.versailles.inra.fr/Species/Botrytis/Sequences-Databases